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Hua-Xin [US/US]; 4307 Forest Edge Trail, Durham, North Carolina 27705 (US). YU, Jae-Sung [KR/US]; 623 Exchange Place, Durham, North Carolina 27713 (US). HO-VAV, Avi-Hai [IL/US]; 80 Pleasant Street, #34, Brookline, Massachusetts 02446 (US).

(74) Agents: ARNOLD, Craig, J. et al.; Amster, Rothstein & Ebenstein LLP, 90 Park Avenue, New York, NY 10016 (US).

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(71) Applicants (for all designated States except US): ALBERT EINSTEIN COLLEGE OF MEDICINE OF YESHIVA UNIVERSITY [US/US]; 1300 Morris Park Avenue, Bronx, New York 10461 (US). BETH ISRAEL DEACONESS MEDICAL CENTER, INC. [US/US]; 330 Brookline Avenue, Boston, Massachusetts 02215 (US). DUKE UNIVERSITY [US/US]; University Office of Science and Technology, 454 Davison Building, Trent Drive, Box 3664 DUMC, Durham, North Carolina 27710 (US).

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(54) Title: MYCOBACTERIA EXPRESSING HIV-1 AND MALARIA ANTIGENS

(57) Abstract: Provided are recombinant mycobacteria expressing an HIV-1 antigen and a malarial antigen. Also provided are Mycobacterium smegmatis expressing an HIV-1 antigen. Further provided are vaccines capable of inducing an immune response in a mammal against HIV-1 and the malarial pathogen. Additionally provided are methods of inducing an immune response in a mammal against HIV-1 and a malarial pathogen. Also provided are methods of inducing an immune response in a mammal against HIV-1. The methods comprise infecting the mammal with any of the above-described mycobacteria.

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MYCOBACTERIA EXPRESSING HIV-1 AND MALARIA ANTIGENS

CROSS-REFERENCE TO RELATED APPLICATION

- 5 This application claims the benefit of U.S. Provisional Application No. 60/643,536, filed January 12, 2005.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

- 10 The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided by the terms of Grants No. AI45705 and AI52816, awarded by the National Institutes of Health.

BACKGROUND OF THE INVENTION

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30

There remains an urgent need for an effective malaria vaccine. Based on previous studies demonstrating that immunization with Msp1 protein can protect Aotus monkeys from severe forms of malaria, the demonstration that Msp-1 can be highly expressed in BCG and elicit antibody bodes well for a recombinant BCG vaccine to minimally prime for an effective malarial
35 response.

There is also an urgent need for an HIV vaccine. Recombinant attenuated non-pathogenic mycobacteria expressing HIV immunogens are attractive vaccine candidates because of the proven safety and immunogenicity of *Mycobacterium bovis* BCG in humans as a vaccine against tuberculosis.

5 An effective HIV/AIDS vaccine will likely need to elicit virus-specific neutralizing antibodies and cytotoxic T lymphocyte (CTL) responses. Although an immunogen that induces antibodies that neutralize a diversity of primary HIV-1 isolates has not yet been defined, a number of strategies are being developed for generating HIV-1-specific CTL (Letvin, 2002). However, there are problems associated with each of these approaches for eliciting CTL that will likely limit
10 their ultimate effectiveness. Plasmid DNA has not proven nearly as immunogenic in humans as it has in laboratory animals (Calarota et al., 1998; MacGregor et al., 1998; Wang et al., 1998). The immunogenicity of replication-defective adenovirus serotype 5 is limited in human populations by pre-existing serotype-specific anti-adenovirus antibodies (Molnar-Kimber et al., 1998). Pox-vectored vaccines only elicit very short-lived immunity in humans (Andrew McMichael, personal
15 communication) and production problems have slowed the development of alphavirus-based vaccine vectors (Weiss and Schlesinger et al., 1991). Better vector systems will therefore be needed to induce anti-HIV-1 cellular immunity and prime for broadly neutralizing antibody responses.

 Mycobacteria have features that make them attractive as potential HIV-1 vaccine vectors.
20 They can be readily engineered to stably express transgenes and can elicit long-lasting cellular and mucosal immune responses (Lagranderie et al., 1997; Mederle et al., 2002). Most importantly, they have been used successfully as vaccines. The attenuated, non-pathogenic *Mycobacterium bovis* BCG is widely used as a vaccine for tuberculosis (TB) and leprosy (Huebner, 1996; Kulkarni and Zodpey, 1999). Recombinant BCG (rBCG) vaccine constructs
25 have shown immunogenicity and protection in murine models against various infectious agents, including *Borrelia burgdorferi*, *Streptococcus pneumoniae*, *Bordetella pertussis*, rodent malaria, leishmania, and measles virus (Connell et al., 1993; Fennelly et al., 1995; Langermann et al., 1994; Matsumoto et al., 1998; Nascimento et al., 2000; Stover et al., 1993). In murine and monkey studies, we and others have shown that rBCG-elicited antibody and cell-mediated
30 responses against HIV-1 and SIV antigens (Ahmad-Nejad et al., 2002; Honda et al., 1995; Stover et al., 1991; Yasutomi et al., 1993).

Mycobacterium smegmatis has a number of properties that may make it an effective vaccine vector. Some *M. smegmatis* strains are non-pathogenic and commensal in humans (Bange et al., 1999; Newton et al., 1993; Pierre-Audigier et al., 1997). Unlike other
35 mycobacterial species such as BCG that survive in host cells for months by inhibiting phagosome

maturation, *M. smegmatis* is rapidly destroyed by phagolysosomal proteases in the phagosomes of infected cells (Kuchnel et al., 2001; Luo et al., 2000; Via et al., 1997; 1998). Nevertheless, *M. smegmatis* can induce cytokine production by macrophages better than pathogenic mycobacterial species (Beltan et al., 2000; Yadev et al., 2004), and can activate and induce the maturation of dendritic cells better than BCG by upregulation of MHC I and costimulatory molecules (Cheadle et al., 2005). *M. smegmatis* can also access the MHC class I pathway for presentation of mycobacterial antigens more efficiently than BCG (Neyrolles et al., 2001). The present studies were initiated to assess the ability of recombinant *M. smegmatis* to elicit HIV-1 envelope-specific CD8+ T cell responses.

Since malaria & HIV coexist in developing and underdeveloped countries, there is a need for inexpensive vaccines for either pathogen. There is also a need for more effective malaria and HIV vaccines. The present invention addresses those needs.

SUMMARY OF THE INVENTION

The present invention is directed to recombinant mycobacteria expressing an HIV-1 antigen and a malarial antigen.

Additionally, the invention is directed to vaccines comprising any of the above-identified mycobacteria, where the mycobacteria are capable of inducing an immune response in a mammal against HIV-1 and the malarial pathogen.

The invention is further directed to methods of inducing an immune response in a mammal against HIV-1 and a malarial pathogen, the methods comprising infecting the mammal with any of the above-described mycobacteria.

The invention is also directed to *Mycobacterium smegmatis* expressing an HIV-1 antigen.

The invention is additionally directed to methods of inducing an immune response in a mammal against HIV-1. The methods comprise infecting the mammal with any of the above-described mycobacteria.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph showing the antibody response in Balb/c mice immunized with rBCG expressing PfMSP1-19.

FIG. 2 is a blot showing the expression of PfMSP1-19::P2P30 in rBCG.

FIG 3 is a graph showing the antibody response in Balb/c mice immunized with rBCG expressing PfMSP1-19.

FIG 4 is a graph showing the T cell response in Balb/c mice immunized with rBCG expressing PfMSP1-19.

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FIG. 5 is a vector diagram and a photograph of a western blot that shows the expression of HIV-1 HXBc2 gp120 envelope in the non-pathogenic *Mycobacterium smegmatis*. Panel A is a diagram showing a codon-optimized *HXBc2 gp120 env* that was cloned into the *E. coli*/mycobacteria shuttle plasmids pJH222 (multicopy) and pJH223 (integrative). The *gp120 env* in the plasmids is under the *M. tuberculosis (Mtb) α-Ag* promoter. A fusion protein was created in which the *M. tuberculosis* 19-kDa signal sequence was at the N-terminus and an influenza hemagglutinin epitope (HA) tag was at the C-terminus of the gp120 Env. Both plasmids contained the Tn903-derived *aph* gene conferring kanamycin-resistance as a selectable marker, and an *E. coli* origin of replication (*oriE*). The origin of replication (*oriM*) was inserted into the pJH222 plasmid, while the *attP* site and the *int* gene of mycobacteriophage L5 were included in pJH223. Panel B shows the results of a western blot analysis showed expression of the gp120 protein in recombinant *M. smegmatis* MC²155. The gp120 expression of three independent clones of mycobacteria transformed with either pJH222-*gp120* (lanes 1-3) or pJH223-*gp120* (lanes 4-6) was determined using an anti-HA mAb (clone 3F10). Mycobacteria transformed with either mock pJH222 or pJH223 containing an irrelevant gene (malaria *msp1*) were utilized as negative controls (lanes 7 and 8).

FIG. 6 is graphs of experimental results showing that recombinant *M. smegmatis* elicited HIV-1-specific CD8 T-cell responses in mice. Balb/c mice were inoculated via the intraperitoneal route with approximately 10⁶ CFU or 10⁸ CFU gp120-expressing recombinant *M. smegmatis* (rSmeg-gp120) transformed with either the integrative pJH223-*gp120* plasmid (Panel A) or the multi-copy pJH222-*gp120* (Panel B). As a negative control, mice were inoculated with the same dose of mycobacteria transformed with the control pJH222- and pJH223-*msp1* plasmids (rSmeg control) (Panel C). Mice were inoculated twice (ten weeks apart) with the same dose of either the rSmeg-gp120 (integrative) construct or the rSmeg control. The mean (± SEM) percent HIV-1 HXBc2 gp120 p18-tetramer positive CD8 T cells from PBMC collected at the indicated time points are shown for each group of mice (n=4 per group).

FIG. 7 is graphs showing *in vitro* function of HIV-specific CD8 T cells primed by r*M. smegmatis*. Panel A is a graph showing cytotoxic activity of HIV-1-specific CD8+ T cells elicited by r*M. smegmatis* immunization. HIV-1-specific CTL were expanded *in vitro* by stimulating splenocytes isolated from mice at day 14 after a single inoculation with 10⁸ CFU r*M. smegmatis* expressing gp120 (integrative) with 10 ng/ml p18 peptide in the presence of rat IL-2 for 7 days. Cytotoxic activity of the effector cells for P815 target cells pulsed with or without p18 was assessed in a ⁵¹Chromium release assay. Effector to target (E:T) ratios used in the study are indicated. Panel B is a graph showing r*M. smegmatis*-elicited HIV-1-specific CD8+ T cells secreted IFN-γ. Day 7 splenocytes from mice immunized with 10⁷ CFU recombinant

mycobacteria expressing gp120 (integrative) were exposed to no peptide, p18, or a gp120 peptide pool, and evaluated in an ELISPOT assay. Splenocytes from mice immunized with r*M. smegmatis* expressing Msp1 were used as a control. The mean (\pm SEM) spot-forming cells (SFC) per 10^6 splenocytes for each group of mice (n=4 per group) is shown.

5 FIG. 8 is graphs showing the phenotype of HIV-1-specific CD8 T cells elicited by immunization with r*M. smegmatis*. Mice were immunized with 10^8 CFU r*M. smegmatis* expressing gp120 (integrative). Panel A shows a flow cytometric analysis of week one PBMC and splenocytes from immunized mice revealed expression of CD44 on the surface of all r*M. smegmatis*-elicited tetramer+ cells. CD62L and CD127 were expressed on a subset of the
10 tetramer+ cells. Panel B shows the proportions of effector (P18-tetramer+, CD127-, and CD62L^{lo}), effector memory (P18-tetramer+, CD127+, and CD62L^{lo}), and central memory (P18-tetramer+, CD127+, and CD62L^{hi}) cells in the blood and spleen of mice immunized with r*M. smegmatis*. Effector, effector memory, and central memory cells are denoted as E, EM, and CM, respectively. The mean (\pm SEM) percent E, EM or CM for each group of mice (n=4 per group) is
15 shown. Panel C shows that peripheral blood HIV-1-specific CD8+ T cells from mice one year after immunization with r*M. smegmatis* expressing gp120 were predominantly central memory cells. PBMC were pooled from 4 mice that were inoculated twice (ten weeks apart) with 10^8 CFU bacilli.

FIG. 9 is a graph showing that recombinant *M. smegmatis*-elicited HIV-1-specific CD8+
20 T cell responses in mice pre-immunized with BCG. Mice were immunized with wildtype BCG (Pasteur) or PBS and six months later with 10^8 CFU r*M. smegmatis* expressing gp120 (integrative) (indicated as BCG + rSmeg-gp120 and PBS + rSmeg-gp120, respectively). BCG-pre-immunized mice that were subsequently inoculated with rSmeg control were used as a negative control (indicated as BCG + rSmeg control). Tetramer analysis was performed on the
25 PBMC of mice one week after inoculation with the r*M. smegmatis* constructs. The mean (\pm SEM) percent of HIV-1 HXBc2 gp120 P18-tetramer positive CD8+ T cells is shown for each group (n=4-5 mice per group).

FIG. 10 is a cartoon showing the strategy to express HIV *env* in BCG or attenuated MTB.

FIG. 11 is a blot showing the expression of HIV-1 CON6-gp120 and CON6-gp140CF
30 envelope proteins in *M. smegmatis*. The expression of intact CON6 gp120 was demonstrated in *M. smegmatis* transformed by the surface expression plasmid of pJH152 (lane 4), the intracellular expression plasmid of pJH153 (lane 5), and the secreted expression plasmid of pJH154 (lane 6). The expression of CON6 gp140CF was also performed in *M. smegmatis* transformed by the surface expression plasmids such as pJH152 (lane 7) and pJH222 (lane 8). Both the intact and
35 partially cleaved gp140 products were shown as demonstrated using gp120 mab T8 (anti-C1

gp120 region), gp41 specific mab 7B2, and V3-loop specific 7B9. Theoretical molecular mass based on amino acids sequences of CON6 gp120 without glycosylation is approximately 53 and and CON6 gp140CF is 71 kDa. However, the protein band of CON6 gp120 and CON6 gp140CF expressed in *M. smegmatis* is approximately 70 kDa and 80kDa, respectively. This suggested that the expression of both CON6 gp120 and CON6 gp140CF in *M. smegmatis* is only partially glycosylated.

FIG. 12 is a graphic showing the binding location of HIV-1 Mabs on HIV-1 CON6 envelope proteins. The figure shows the full-length gp140 and its reacting sites of T8, 7B2, and 7B9. However, in the cleaved gp140, 30 kDa peptide only reacts with T8 while 50 kDa peptide reacts with both 7B2 and 7B9. Thus this data suggests that 50kDa cleaved peptide contains V3 – loop region and gp41.

FIG. 13 shows the *r-M. smegmatis* immunization schedule in mice. We set up total 60 group of mouse immunization study with *M. smegmatis* and HIV ENV constructs in mycobacterial with 5 mice per group. Mice were immunized 8 different immunogens by the two different injection route: intraperitoneal (IP) vs intradermal (ID). The immunogens were such as controls (*M. smegmatis*, Empty pJH222/*M. smegmatis*), HIV envelope gp120 (pJH152-gp120/*M. smegmatis*, pJH153-gp120/*M. smegmatis*, pJH154-gp120/*M. smegmatis*), and HIV envelope gp140CF (pJH152-gp140CF/*M. smegmatis*, pJH222/*M. smegmatis*, pJH154-gp140CF/*M. smegmatis*). Each immunogen was immunized with four different dosage groups such as 10^{10} CFU, 10^9 CFU, 10^8 CFU, and 10^7 CFU. After the initial immunization, we have bled mice every two weeks and antibodies responses were monitored by ELISA. Boosting with the same immunogens was done in week 10 and with CON6-gp140CF in week 15.

FIG. 14 shows ELISPOT wells after stimulation with MTB whole cell lysate or saline. Mice were single IP injection of 10^9 CFU pJH152-gp140CF/*M. smegmatis*. After two weeks IP immunization, harvest mice spleens for IFN γ ELISPOT using native MTB antigens from NIAID-funded reagent program at Colorado State University.

FIG. 15 is a graph of mycobacterial antigen-specific IFN γ spot-forming cells after a single IP inoculation with recombinant *M. smegmatis* or medium. This figure shows MTB WCL could be better choice for IFN γ ELISPOT rather than MTB CFP, MTB antigen 85, or PPD. (Native mycobacterial antigens: CFP, culture filtrate protein; WCL, whole cell lysate; Ag 85, antigen 85 complex; PPD, purified protein derivative).

FIG. 16 is a western blot and a diagram showing amounts of malaria antigen produced by various *M. smegmatis* clones having plasmid constructs containing one of two fragments of the *P. falciparum* MSP antigen gene.

FIG. 17 is graphs showing cytokine expression by gp120-specific CD4⁺ T cells. Mice were immunized with Smeg-gp120 (5×10^7 CFU), Vac-gp160 (2×10^7 PFU), Ad-gp140 (2×10^7 particles) or DNA-gp120 (50 μ g) and 10 weeks later were boosted with the same quantity of vector used for priming. Splenocytes were harvested one week (Vac-gp160 and Smeg-gp120) or two weeks (Ad-gp140 and DNA-gp120) after the prime and boost immunization, or 10 weeks after the prime immunization (pre-boost). The cells were cultured for 6 hr in the presence of medium alone or a pool of 47 overlapping peptides spanning the HIV-1 III_B gp120 protein (2 μ g/ml). Data are presented as the percentages of CD4⁺ IFN- γ ⁺ and CD4⁺ IL-2⁺ T cells following peptide stimulation and represent the means of five mice per group \pm SE.

FIG. 18 is graphs showing results from heterologous prime-boost immunization using Smeg-gp120 or DNA-gp120 followed by Ad-gp140. Mice were immunized with Smeg-gp120 (5×10^7 CFU) or DNA-gp120 (50 μ g) and 10 weeks later some mice were similarly immunized a second time. Panel A shows expression of CD62L, CD127 and CD27 on p18-specific CD8⁺ T cells 20 weeks after the first immunization ($\times 1$) and 10 weeks after the second immunization ($\times 2$). Panel B shows the kinetics of p18-specific CD8⁺ T cells in Smeg-gp120 and DNA-gp120 immunized mice (prime and boost) following heterologous immunization with 10^6 particles of Ad-gp140. Data represent the mean of 5-10 mice per group \pm SE.

FIG. 19 is graphs showing a functional analysis of the p18-specific CD8⁺ T cells and CD4⁺ T cells elicited by priming with Smeg-gp120 or DNA-gp120 followed by boosting with Ad-gp140. Mice were immunized either once ($\times 1$) or twice ($\times 2$), 10 weeks interval, with Smeg-gp120 (5×10^7 CFU) or DNA-gp120 (50 μ g). 20 weeks after the first immunization and 10 weeks after the second immunization the mice were inoculated with 10^6 particles of Ad-gp140. Splenocytes were harvested 8 weeks after the immunization with Ad-gp140 and were cultured for 6 hr in the presence of medium alone, p18 peptide (2 μ g/ml) or Env peptide pool (1 μ g/ml). Intracellular production of IFN- γ and IL-2 by CD8⁺ p18-specific T cells and CD4⁺ T cells or CD107a/b expression by p18-specific CD8⁺ T cells were evaluated. Data are presented as the percentages of tetramer positive CD8⁺ T cells staining positively for IFN- γ , IL-2 or CD107a/b, and CD4⁺ T cells staining positively for IFN- γ or IL-2 and represent the means of five mice per group \pm SE.

FIG. 20 is graphs showing IFN- γ production by CD4⁺ and CD8⁺ T cells following priming immunization with Smeg-gp120 or DNA-gp120, and boosting immunization with Ad-gp140. Mice were immunized either once ($\times 1$) or twice ($\times 2$), 10 weeks apart, with Smeg-gp120 (5×10^7 CFU) or DNA-gp120 (50 μ g). Twenty weeks after the first immunization and 10 weeks after the second immunization the mice were inoculated with 10^6 particles of Ad-gp140. Splenocytes were harvested from individual mice 8 weeks after the immunization with Ad-gp140.

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IFN- γ productions were evaluated by ELISPOT assay using total splenocytes incubated with p18 peptide (1 μ g/ml) (Panel A) or by CD8⁺ depleted splenocytes stimulated with peptide pool consisted of 158 overlapping 15-mer peptides spanning the HIV-1 HXB2/BaL Env protein at a concentration of 1 μ g/ml (Panel B). Data are presented as the mean number of antigen-specific spot per 10⁶ spleen cells \pm SE with five mice per group.

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to recombinant mycobacteria expressing an HIV-1 antigen and a malarial antigen. Since these mycobacteria are usually used *in vivo*, it is preferred that the mycobacteria is avirulent or rendered so, e.g., by selecting for avirulent strains or by engineering the mycobacteria to have a mutation or mutations that can fulfill that purpose. Many such mutations are known in the art, for example mutations that render the mycobacterium auxotrophic, e.g., a *pan* mutation or a *Lys* mutation, or mutations eliminating pathogenicity genes such as an *RDI* deletion, as is known in the art. It is also preferred that the mycobacterium utilized for this invention can colonize the host, in order for the mycobacterium to provide a long term antigenic stimulus to the host, thus establishing a strong immune response. Non-limiting examples of useful mycobacteria are *Mycobacterium smegmatis*, *Mycobacterium bovis*-BCG, *Mycobacterium avium*, *Mycobacterium phlei*, *Mycobacterium fortuitum*, *Mycobacterium lufu*, *Mycobacterium paratuberculosis*, *Mycobacterium habana*, *Mycobacterium microti*, *Mycobacterium scrofulaceiu*, *Mycobacterium intercellulare*, *Mycobacterium tuberculosis*, and any genetic variant thereof. Preferably, the mycobacterium is a *Mycobacterium smegmatis*, a *Mycobacterium bovis*-BCG, or an attenuated *Mycobacterium tuberculosis*, since those strains have been extensively studied and established to be avirulent, but able to establish a long term colonization of the host. A particularly preferred strain is *Mycobacterium smegmatis* MC²155 or a derivative thereof.

Any HIV-1 antigen that can establish an immune response in the host is useful for the present invention. Examples include gp120 env, gp140 env, gp160 env, gag, pol, vif, vpr, vpu, tat, rev and nef. A preferred HIV-1 antigen is gp120 env.

Similarly, the invention is not limited to any particular malarial antigen. Preferably, the antigen is sufficiently immunogenic to establish a useful anti-malarial immune response. The antigen can be from any malarial parasite, including *Plasmodium falciparum*, *P. yoelii*, and *P. knowlesi*. Examples of useful malarial antigens include the merozoite surface protein-1 (MSP-1) protein from *Plasmodium falciparum*, the blood stage antigen AMA-1, and the circumsporozoite antigen CSP-1. Particularly preferred is an MSP antigen. See Example 1.

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Preferably, the mycobacterium is *M. smegmatis*, the viral antigen is HIV-1 gp120 or HIV-1 gp140 (*env* antigens), and the malarial antigen is MSP-1, since the examples (below) show that this combination establishes a strong immune response. More preferably, the malarial antigen is the MSP-1 antigen PfMSP1-19, which is the C-terminal fragment of MSP-1. Most preferably, the PfMSP1-19 antigen further comprises a portion of the PfMSP1.42 fragment that comprises most of the MSP1.33 fragment. See Example 3.

As shown in Example 1, the GC content of the gene encoding an expressed recombinant protein can affect the expression level of the protein in mycobacteria, since mycobacteria prefer genes with a high GC content. Since many malarial antigens, in particular the MSP-1 antigen, has a high AT content, engineering the gene encoding the malarial antigen to have increased GC content is effective at increasing expression of the antigen from the mycobacteria, and also increases the ability of the mycobacteria to elicit an immune response to the malaria antigen. Thus, preferably the malarial antigen is expressed from a gene that has been engineered to have an increased GC content.

It is sometimes desirable that the mycobacterium further comprises a reporter gene that is expressed when the mycobacterium infects a mammalian host, such that expression of recombinant genes from the mycobacterium can be monitored, quantified and/or localized. These embodiments are not narrowly limited to any particular reporter gene, and the skilled artisan could determine a reporter gene for any particular purpose. In some preferred embodiments, the reporter gene is a fluorescent protein or a detectable antigen.

The mycobacteria of the present invention can also utilize any promoter to drive the expression of the HIV-1 and malarial antigens. Preferred promoters are the mycobacterial promoters *hsp60*, *mtrA*, *18kD*, *α -Ag*, and *aceA*, since those promoters are known to provide sufficient expression to induce an immune response to the antigen in the host. Most preferably, the mycobacterial promoter for the HIV-1 antigen and the malarial antigen is *α -Ag*.

As shown in Example 1, it is also preferred if the HIV-1 antigen and the malarial antigen is expressed from genes further encoding a signal sequence that facilitates expression of the viral protein in the mycobacterial membrane. Most preferably, this signal sequence is the 19-kDa signal sequence.

Most preferably, the mycobacterium of these aspects of the invention is *M. smegmatis* MC²155 or a derivative thereof; the HIV-1 antigen is gp120 *env*; the malarial antigen is MSP-1; the HIV-1 antigen and the malarial antigen is expressed from genes further encoding the 19-kDa signal sequence; and the mycobacterial promoter for the HIV-1 antigen and the malarial antigen is *α -Ag*.

The present invention is also directed to *Mycobacterium smegmatis* expressing an HIV-1 antigen. Preferably, the *M. smegmatis* is *M. smegmatis* MC²155 or a derivative thereof. The HIV antigen of these *M. smegmatis* is preferably gp120 env, gp140 env, gp160 env, gag, pol, vif, vpr, vpu, tat, rev or nef; most preferably gp120 env. It is also preferred if the HIV-1 antigen is expressed from a gene further encoding the 19-kDa signal sequence, and a mycobacterial promoter for the HIV-1 antigen is α -Ag.

Most preferably, the *Mycobacterium smegmatis* is *M. smegmatis* MC²155 or a derivative thereof; the HIV-1 antigen is gp120 env; the HIV-1 antigen is expressed from a gene further encoding the 19-kDa signal sequence; and the mycobacterial promoter for the HIV-1 antigen is α -Ag.

The invention is also directed to a vaccine comprising a recombinant mycobacterium expressing an HIV-1 and a malarial pathogen as described above, where the mycobacterium is capable of inducing an immune response in a mammal against HIV-1 and the malarial pathogen. Preferably, the mammal is a human.

The present invention is additionally directed to methods of inducing an immune response in a mammal against HIV-1 and a malarial pathogen. The methods comprise infecting the mammal with one of the above-described mycobacteria that expresses an HIV-1 and a malarial pathogen. Several different inoculation methods are known for infecting the mammal sufficiently to establish a long-term colonization of the mammalian host. The skilled artisan can determine without undue experimentation the most useful method of for any particular purpose.

In preferred embodiments of these methods, the mammal is a human.

The invention is further directed to methods of inducing an immune response in a mammal against HIV-1. The methods comprise infecting the mammal with any of the above-described mycobacteria.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims, which follow the examples.

Example 1. Optimizing *Mycobacterium bovis* BCG malaria vaccines.

Recombinant *Mycobacterium bovis* BCG constructs were created to optimize malarial antigen expression that elicits priming or protective immune responses to malarial antigens. BCG has the potential to deliver foreign antigens to children at birth in a developing world setting.

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Moreover, based on its persistence and immunogenicity, it has the potential to elicit long-lasting immune responses.

Results

In order to test the hypothesis that rBCG strains expressing a higher level of recombinant malaria surface antigen PfMSP1-19 will be more immunogenic in mice, the PfMSP1-19 gene sequence was cloned into a mycobacterium expression vector under the control of various mycobacterial promoters including hsp60, mtrA, 18 kD, a-Ag, and aceA. PfMSP1-19 was also expressed and directed to different locations within and associated with the rBCG bacilli including intracellular, secretory, and surface locales. The codons of the PfMSP1-19 gene were also changed from the wildtype malaria sequence that is AT-rich to GC-rich. The expression was highest in rBCG expressing GC-rich PfMSP1-19 gene under the control of α -Ag on the cell surface of the mycobacteria among the constructs tested (Table 1).

Table 1. Recombinant BCG expressing PfMSP1-19 from AT-rich and GC-rich gene sequences.

AT-RICH					GC-RICH				
PROMOTER	S.S	Plasmid	Expression	Antibody	PROMOTER	S.S	Plasmid	Expression	Antibody
hsp		94	-	-	hsp 19 KD	135	++++	++++	
hsp 19 KD	95	+	-	-	hsp	136	+	-	-
hsp a-Ag	96	-	-	-	hsp a-Ag	137	-	-	-
mtrA	97	-	-	-	mtrA 19 KD	140	++	+++	
mtrA 19 KD	98	+	-	-	mtrA	141	-	-	-
mtrA a-Ag	99	-	-	-	mtrA a-Ag	142	-	-	-
18kD	100	-	-	-	18kD 19 KD	143	++	-	-
18kD 19 KD	101	-	-	-	18kD	144	-	-	-
18kD a-Ag	102	+	++		18kD a-Ag	145	-	-	-
aceA	103	-	-	-	aceA 19 KD	146	-	-	-
aceA 19 KD	104	-	-	-	aceA	147	-	-	-
aceA a-Ag	105	-	-	-	aceA a-Ag	148	-	-	-
Pa-Ag a-Ag	106	-	-	-	Pa-Ag a-Ag	158	-	-	-
P19kD 19KD	107	-	-	-	P19kD 19KD	159	-	-	-
amd	108	-	-	-	amd 19 KD	149	+	-	-
amd 19 KD	109	-	-	-	amd	150	-	-	-
amd a-Ag	110	-	-	-	amd a-Ag	151	-	-	-
Pa-Ag	*121	-	-	-	Pa-Ag 19 KD	152	++++	++++	
Pa-Ag 19 KD	122	+	-	-	Pa-Ag	153	+	-	-
Pa-Ag a-Ag	123	-	-	-	Pa-Ag a-Ag	154	+	-	-
P19kD	*125	-	-	-	P19kD 19 KD	155	-	++	
P19kD 19 KD	126	-	-	-	P19kD	156	-	-	-
P19kD a-Ag	127	-	-	-	P19kD a-Ag	157	-	-	-

The ability of these rBCG strains to generate antibody response in Balb/c mice was then tested. A correlation between the level of antibody generated in mice immunized with rBCG and the amount of protein expressed in rBCG was found (FIG.1 and Table 1). rBCG (B152) expressed the highest level of PfMSP1-19 and induced the highest level of antibody response in Balb/c mice. In subsequent studies, we tested the immunogenicity of rBCG (B152) in different strains of mice including Balb/c, C57B/L 6, C3H, and Swiss mice.

P2P30 T cell epitopes from tetanus toxin have been reported to act as adjuvant that enhances the T cell response in mice when it was fused to various antigens (Panina-Bordignon et al., 1989). The hypothesis that P2P30 universal T cells epitopes enhances the T cells response to malaria surface antigen PfMSP1-19 was tested in mice. The epitope sequences in the 3' end of the malaria antigen PfMSP1-19 gene was cloned. The expression of the fusion protein in rBCG (B103) appeared to be equivalent to that of rBCG (B152) without the epitope (FIG. 2).

The effect of P2P30 T cell epitopes for its immunogenicity in mice by immunizing Balb/c mice with rBCG (B103) subcutaneously or intraperitoneally was first tested. The blood samples were collected at 14 weeks post immunization. The level of antibody against bv-PfMSP1-42 was then measured. Fourteen (14) weeks after immunization, the antibody level of serum from mice immunized with rBCG (B152) was significantly higher than mice immunized with control BCG carrying only empty vector rBCG (B216), though intraperitoneal route of immunization seemed to induce more antibody response than subcutaneous route, confirming earlier observations. While rBCG (B103) also induced significant antibody response in mice, the presence of P30P2 epitopes did not seem to enhance the antibody response to PfMSP1-42 antigen. The immunogenicity of recombinant *M. smegmatis* expressing very high level of PfMSP1-19 (see recombinant *M. smegmatis* S8) was also compared to those of recombinant BCG. Interestingly, at week 14, mice receiving the same dose of recombinant *M. smegmatis* generated a similar level of antibody to those mice receiving rBCG (B152) (FIG. 3).

The T cell response in the same groups of mice at 14 weeks post-immunization by T cell proliferation assays was also characterized (FIG. 4). Splenocytes (2×10^5) of three mice from each group were collected and incubated with either 1 or 10 mg of rPfMSP1-19 protein for 2 days and uptake of tritiated thymidine by splenocytes was measured. A Stimulation Index was calculated by dividing the thymidine intake of splenocytes that were stimulated with rPfMSP1-19 protein antigen by splenic cells that were not stimulated. FIG. 3 shows that the T cell response was higher (5-10 fold increase in Stimulation Index) in mice immunized subcutaneously with rBCG expressing PfMSP1-19 (B152) than mice immunized with rBCG carrying empty vector (B216). Subcutaneous immunization of rBCG expressing PfMSP1-19 generated higher T cell response than intraperitoneal immunization. However, P30P2 epitope fusion seemed to have a negative effect on T cell stimulation in mice immunized subcutaneously or intraperitoneally with rBCG expressing PfMSP1-19. Interestingly, at week 21 post immunization, the group of mice receiving recombinant *M. smegmatis* expressing high levels of PfMSP1-19 generated a higher Stimulation Index (2 fold) than mice receiving recombinant BCG expressing PfMSP1-19.

Example 2. Generation of CD8+ T cell Responses by a Recombinant Nonpathogenic
Mycobacterium smegmatis Vaccine Vector Expressing HIV-1 Env.

Example summary

Because the vaccine vectors currently being evaluated in human populations all have
5 significant limitations in their immunogenicity, novel vaccine strategies are needed for the
elicitation of cell-mediated immunity. The nonpathogenic, rapidly growing mycobacterium *M.*
smegmatis was engineered as a vector expressing full length HIV-1 HXBc2 envelope protein.
Immunization of mice with recombinant *M. smegmatis* led to the expansion of MHC class I-
10 restricted HIV-1 epitope-specific CD8+ T cells that were cytolytic and secreted IFN- γ . Effector
and memory T lymphocytes (CTL) were elicited, and repeated immunization generated a stable
central memory pool of virus-specific cells. Importantly, pre-existing immunity to BCG had only
a marginal effect on the immunogenicity of recombinant *M. smegmatis*. This mycobacterium
may therefore be a useful vaccine vector.

Materials and Methods

15 Generation of recombinant mycobacteria. *Mycobacterium smegmatis* MC²155 was
grown in Middlebrook 7H9 (Difco) supplemented with 10% ADS and 0.05% Tween 80 (Fisher
Scientific). *Mycobacterium bovis* BCG (Pasteur) was grown in 7H9 media supplemented with
10% OADC (Difco) and 0.05% Tween 80. A human codon-optimized HIV-1 III_B gp120
envelope gene (HXBc2) was cloned into the multi-copy pJH222 and single copy integrative
20 pJH223 *E. coli*/mycobacteria shuttle plasmids. A synthetic operon was constructed containing the
viral envelope gene, which is regulated by the *M. tuberculosis* α -antigen promoter and the *M.*
tuberculosis 19-kDa signal sequence. For detection of the HIV-1 envelope protein, an HA-tag
was fused to the C-terminal end of the envelope. Within the operon, a kanamycin resistance gene
was cloned downstream of the viral gene. The multi-copy and integrative plasmids with the
25 HXBc2 envelope gene insert were transformed into the *M. smegmatis* MC²155 strain.
Recombinant mycobacterial clones were selected for kanamycin resistance on 7H10 agar
containing 20 μ g/ml of kanamycin (Sigma). Single colonies were grown in 7H9 medium
containing 20 μ g/ml of kanamycin and grown by shaking for 2-3 days until an OD₆₀₀
approximately equal to 1. Mycobacteria were then harvested and washed twice in ice cold PBS.
30 Expression of the viral gp120 protein was assessed by Western blotting of mycobacterial lysates
(1 μ g of total protein) using an anti-HA mAb (clone 3F10) and a chemiluminescence detection
kit, according to the manufacturers protocol (Roche Applied Science).

Mice and immunizations. 8-12 week old female Balb/c mice were purchased from
Taconic and Charles River laboratories. Mice were housed in a biosafety level 3 facility under
35 specific pathogen-free conditions at the Center for AIDS Research Animal Biohazard

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Containment Core Suite (Dana-Farber Cancer Institute). Research on mice was approved by the Dana-Farber Cancer Institute Animal Care and Use Committee. Recombinant *M. smegmatis* and BCG were grown in 7H9 medium until an OD₆₀₀ approximately equal to 1. We estimated that bacterial growth to an OD value of 1 is equal to 5×10^8 colony-forming units (CFU). For *rM. smegmatis* immunizations, approximately 10^6 or 10^8 CFU bacilli were injected via the intraperitoneal route (i.p.) in 200 μ l of sterile PBS, 0.02% Tween. Approximately 10^6 CFU bacilli were injected subcutaneously (s.c.) for BCG immunization.

Tetramer staining and flow cytometric analysis. H-2D^d tetrameric complexes folded with the P18-peptide (RGPGRAFVTI) (Takahashi et al., 1992), a sequence found in the V3 loop of HIV-1 HXBc2 envelope protein, was prepared as described previously (Staats et al., 2001). Mice were anesthetized with Isoflurane and bled retro-orbitally. Blood was collected in RPMI 1640 containing 40 U of heparin (American Pharmaceutical Partners) per ml. Peripheral blood mononuclear cells (PBMCs) were isolated using lympholyte-M (Cedarlane) and stained with the P18-tetramer conjugated with phycoerythrin (PE) and anti-CD8 α mAb (Ly-2; Caltag) conjugated with allophycocyanin (APC) to detect P18-specific CD8⁺ T cells. The cells were washed in PBS containing 2% fetal bovine serum (FBS) and fixed with PBS containing 2% formaldehyde (Polysciences). CD8⁺ T cells were analyzed for tetramer staining using two-color flow cytometry on a FACS Array (BD Pharmingen). For phenotyping the P18-specific CD8⁺ T cells, splenocytes and PBMC were sampled one week after immunization of mice with recombinant mycobacteria and stained with anti-CD8 α mAb (53-6.7; BD Pharmingen) conjugated with PerCP-Cy5.5, anti-CD62L mAb (MEL-14; BD Pharmingen) conjugated with APC, anti-CD44 mAb (IM-7; eBiosciences) conjugated with APC-Cy7, anti-CD127 mAb (A7R34; eBiosciences) conjugated with PE-Cy7, and the P18-tetramer conjugated with PE. Multi-color flow analysis was performed using the BD LSRII Cytometer (BD Biosciences) and the FlowJo software (Tree Star).

⁵¹Chromium release assay. Splenocytes were harvested from mice one week after immunization with 10^7 CFU recombinant mycobacteria. The cells were resuspended in RPMI 1640 containing 10% FBS, and cultured in a 24-well plate (8×10^6 /well) with 10 ng of p18 epitope peptide per ml. IL-2 (Sigma) was added to cultures on day 2 to a final concentration of 10 U/ml. On day 7, cells were harvested, washed once, and used as effectors in a ⁵¹Cr release assay with P815 target cells (American Type Culture Collection). P815 cells were cultured overnight in the presence of medium alone or with 100 ng of p18 peptide per ml. Cells (2×10^6) were labeled with 150 μ Ci of ⁵¹Cr for 1 h at 37 °C, washed twice, and added to a 96-well round-bottom plate at 104/well in 100 μ l of 10% RPMI medium. Titrations of effector cells were added to triplicate wells in 100 μ l of medium. Lytic activity was assessed in a four-hour ⁵¹Cr release assay as

previously described (Seaman et al., 2004). Percent specific lysis was calculated as follows: $100 \times (\text{experimental} - \text{spontaneous release}) / (\text{maximum} - \text{spontaneous release})$.

IFN- γ ELISPOT assay. An ELISPOT assay was performed to measure IFN- γ production as previously described (Seaman et al., 2004). Briefly, 96-well Multiscreen HA plates (Millipore) were coated by overnight incubation (100 μ l/well) at 4°C with rat anti-mouse IFN- γ mAb (clone R4-6A2; BD Pharmingen) at 10 μ g/ml in PBS. Splenocytes were harvested from individual mice one week after immunization with 10^7 CFU recombinant mycobacteria. Effector cells were plated in triplicate at 2×10^5 /well in a 100- μ l final volume with medium alone, 4 μ g of p18 epitope peptide per ml, or 4 μ g of Env peptide pool per ml. The pool consisted of 47 overlapping 15-mer peptides spanning the HIV-1 III_B gp120 protein (Centralized Facility for AIDS Reagents, Potters Bar, United Kingdom) and was used such that each peptide was present at a concentration of 4 μ g/ml. After a 24 h incubation at 37 °C, the plates were washed free of cells with PBS-0.05% Tween 20 and incubated overnight at 4 °C with 100 μ l of biotinylated rat anti-mouse IFN- γ mAb (clone XMG1.2; BD Pharmingen) per well at 5 μ g/ml. Plates were washed four times, and 75 μ l of streptavidin-alkaline phosphatase (Southern Biotechnology Associates) was added at a 1/500 dilution. After a 2 h incubation, plates were washed four times and developed with Nitro Blue Tetrazolium-5-bromo-4-chloro-3-indolylphosphate chromogen (Pierce). Plates were analyzed with an ELISPOT reader (Hitech Instruments).

Statistical analysis. Data were expressed as mean \pm SEM. Statistical tests were performed using Student's t test. A P value less than 0.05 was considered significant.

Results

Generation of recombinant *Mycobacterium smegmatis* expressing HIV-1 gp120 envelope protein. The pJH222 and pJH223 *E. coli*/mycobacteria shuttle plasmids were used to express a HIV-1 HXBc2 *env* gene codon optimized for human cell expression. A human codon-optimized HIV-1 *env* was used since the codons used by human cells are similar to those used by mycobacteria (Andersson and Scharp, 1996; de Miranda et al., 2000; Pan et al., 1998). pJH222 is a multi-copy plasmid that replicates episomally in mycobacteria (Snapper et al., 1988); the pJH223 is an integration-proficient plasmid (Lee et al., 1991) that integrates as a single-copy DNA in the mycobacterial genome (FIG. 5A). Both plasmids contained the *M. tuberculosis* α antigen promoter to drive expression of HXBc2 *env*. The HIV-1 protein was fused to an HA tag at the C-terminus. The N-terminus of the HIV-1 envelope was fused to the 19-kDa signal sequence to facilitate expression of the viral protein in the mycobacterial membrane. Fusion with the 19-kDa signal sequence has been shown to increase immunogenicity of a heterologous protein (Stover et al., 1991).

Both *env*-containing plasmids were transformed into the efficient plasmid transformation mutant *M. smegmatis* MC²155 (Snapper et al., 1988). Western blot analysis using an anti-HA mAb showed that recombinant *M. smegmatis* clones transformed with the multi-copy plasmid had higher expression levels of the viral protein than those transformed with the integrating plasmid (FIG. 5B). Control *rM. smegmatis* constructs, which were transformed with either the pJH222 or pJH223 containing the malaria *msp1* gene, did not express the viral antigen. The predicted molecular weight of the envelope protein expressed by the *rM. smegmatis* constructs suggested an absence of the heavy glycosylation seen in the mature gp120 envelope of HIV-1 (Wyatt et al., 1998). These results show the successful expression of the full-length HIV-1 gp120 protein in the rapidly growing, nonpathogenic *M. smegmatis*.

rM. smegmatis immunization elicited functional HIV-1-specific CD8+ T cells. While BCG and *Mycobacterium vaccae* (Hetzel et al., 1998) have been assessed as potential vaccine vectors, other nonpathogenic species of mycobacteria have not been evaluated for this application. We therefore tested the *rM. smegmatis* constructs for their immunogenicity in mice. Immune responses were monitored in Balb/c mice using a tetramer assay, measuring CD8+ T cell responses to the H-2D^d-restricted P18 epitope from the V3 loop of the HXBc2 envelope protein. Both the multi-copy and the single copy *rM. smegmatis* constructs expressing gp120 elicited peripheral blood HIV-specific CD8+ T cell responses in mice immunized intraperitoneally with either 10⁶ or 10⁸ CFU bacilli ($P < 0.05$ versus control groups at weeks one, two, and three post-immunization) (FIGS. 6A and 6B). Higher frequency responses were seen in mice immunized with 10⁸ CFU than those immunized with 10⁶ CFU bacilli. Interestingly, despite significantly lower viral antigen expression by *rM. smegmatis* containing the highly stable single copy plasmid than *rM. smegmatis* containing the multi-copy plasmid (FIG. 5B), the magnitudes of the immune responses elicited by both constructs were comparable. CD8+ T cell responses peaked at week 1, rapidly declined thereafter, and were undetectable in the peripheral blood by week 4.

Mice were inoculated with *rM. smegmatis* expressing gp120 twice, at an interval of 10-weeks, to determine whether the T cell responses could be boosted. P18-specific responses increased in magnitude, and were seen one week after immunization ($P < 0.05$ versus control groups). However, these peak responses were not greater than those seen following a single inoculation (FIG. 6C). Responses in the boosted mice declined thereafter, but remained detectable even one year following the initial immunization (data not shown). These results demonstrate that *rM. smegmatis* is capable of eliciting MHC class I-restricted CD8+ T cells specific for the HIV-1 envelope.

The functional capacity of the *rM. smegmatis*-induced HIV-1 envelope-specific CD8+ T cells was then determined. Splenocytes were harvested one week after immunization of mice

with 10^7 CFU of the single-copy *rM. smegmatis* construct and evaluated in IFN- γ ELISPOT and ^{51}Cr -release assays. The splenocytes secreted IFN- γ after overnight stimulation with the dominant CD8+ T cell P18 epitope peptide or a pool of overlapping peptides spanning the entire gp120 protein ($P < 0.001$ versus control groups) (FIG. 7B). The cytotoxic activity of the *rM. smegmatis*-elicited CD8+ T cells was also assessed. Splenocytes were stimulated with the P18-peptide for one week in the presence of IL-2 and evaluated as effector cells in a ^{51}Cr -release assay. These effector cells were able to kill efficiently P815 target cells pulsed with the P18 peptide ($P < 0.01$ versus control groups at E:T ratios 50:1, 12:1 and 3:1) (FIG. 7A). Thus, the *rM. smegmatis*-elicited HIV-1-specific CD8+ T cells were capable of secreting IFN- γ and mediating cytolytic activity in response to HIV-1 envelope peptide stimulation.

rM. smegmatis immunization elicited both effector and memory HIV-1 Env-specific CD8+ T cells. To further characterize the *rM. smegmatis*-induced CD8+ T cells, we evaluated these Env-specific T cells for their state of maturation and functional commitment by assessing their expression of CD62L, CD127 and CD44 using surface staining with monoclonal antibodies and flow cytometric analysis. Tetramer+ CD8+ T cells were found in both the spleen and the peripheral blood one week after immunization with *rM. smegmatis* expressing gp120. In contrast, HIV-1-specific CD8+ T cells were not generated in mice immunized with the control mycobacteria construct. All the tetramer+ CD8 cells expressed CD44, indicating that they were activated (FIG. 8A). Moreover, the majority of these CD8 T cells were effector cells (tetramer+, CD44^{hi}, CD127-, and CD62L^{lo}), and a small proportion was either effector memory (tetramer+, CD44^{hi}, CD127+, and CD62L^{lo}) or central memory cells (tetramer+, CD44^{hi}, CD127+, and CD62L^{lo}). This was seen both in the peripheral blood and spleen of the immunized mice (FIG. 8B). One year after immunization with *rM. smegmatis*, essentially all of the peripheral blood tetramer+ CD8+ T cells were central memory cells (FIG. 8C). These data indicate that *rM. smegmatis* can generate effector, effector memory and long-lived central memory HIV-specific CD8+ T cells.

rM. smegmatis elicited HIV-1 Env-specific CD8+ T cells in BCG immune mice. A large proportion of the human population has received BCG as a tuberculosis vaccine, and we were concerned that prior BCG exposure might substantially blunt the immunogenicity of a *rM. smegmatis* vaccine. Whether anti-BCG immunity can affect the immunogenicity of *rM. smegmatis* constructs was therefore evaluated in mice. To induce anti-BCG immunity, mice were inoculated with 10^6 CFU wildtype BCG or PBS and 6 months later were inoculated with *rM. smegmatis* expressing gp120 (Gheorghiu et al., 1994). In fact, only a modest reduction in peak CD8+ tetramer+ responses were observed in the BCG-preimmunized mice (FIG. 9). These

results suggest that pre-existing immunity to BCG may have only a marginal effect on the immunogenicity of r*M. smegmatis*.

Discussion

A novel vaccine vector is described here. The vector is a recombinant nonpathogenic *Mycobacterium smegmatis* MC²155 (r*M. smegmatis*) expressing the entire HIV-1 HXBc2 gp120 envelope protein, which was immunogenic in mice. The strain mc²155 is a mutant of *M. smegmatis* (ATCC 607) that is transformable with pAL5000 plasmids at 7 orders of magnitude higher frequency than the parental strain (Snapper et al., 1990). The efficient plasmid transformation phenotype has caused mc²155 to be the surrogate host of choice for the analysis of genes from pathogenic mycobacteria (Converse and Cox, 2005; de Mendonca-Lima, 2001; Wei et al., 2000) and has recently been sequenced by TIGR (<http://www.tigr.org>). Moreover, this strain has been shown to be nonpathogenic following intravenous infections of SCID mice (Bange et al., 1999). We evaluated a variety of mycobacterial promoters and regulatory genes and found that the use of *M. tuberculosis* α antigen promoter and fusion of the transgene with the 19-kDa signal sequence optimized the immunogenicity of the vaccine construct. A number of factors probably contributed to this increased immunogenicity. This configuration clearly enhanced the expression of the HIV-1 gp120 envelope protein in mycobacteria (data not shown). Furthermore, fusion of the gp120 protein with the 19-kDa protein may have created a chimeric lipoprotein that is immunogenic, perhaps because of acylation of the signal sequence (Young and Garbe, 1991). The acylated moiety was found to be important for MHC class I antigen presentation of lipoproteins, perhaps because it facilitates lipoprotein interaction with the Toll-like receptor 2 (TLR2) (Grobe et al., 2002; Neyrolles et al., 2001).

It is interesting to speculate that the immunogenicity of the HIV-1 envelope 19-kDa fusion lipoprotein in the rapidly growing non-pathogenic r*M. smegmatis* mycobacteria may be associated with the inability of this recombinant vector to persist. Although lipoproteins are certainly immunogenic, persistent exposure to lipoproteins can lead to suppression of antigen presentation by macrophages (Noss et al., 2001; Tobian et al., 2003). Both *M. bovis* BCG and *M. tuberculosis* can persist in host cells and inhibit phagosome maturation (Via et al., 1997; 1998). Persisting *M. tuberculosis* and BCG may exert immunosuppressive effects through ligation of lipoproteins to TLRs localized to the phagosome (Ahmad-Nejad et al., 2002; Ozinsky et al., 2000; Pai et al., 2004; Tobian et al., 2003; Underhill et al., 1999). On the other hand, *M. smegmatis* does not inhibit phagosome maturation and is degraded rapidly by phagolysosomal proteases. An explanation for the robust immunogenicity of the r*M. smegmatis* constructs will likely come from parallel studies of the immunogenicity of the HIV envelope chimeric lipoprotein in rBCG and r*M.*

smegmatis as well as an evaluation of the roles of persistence and lipoprotein-TLR interactions in the generation of CD8+ T cell responses elicited by mycobacteria.

rM. smegmatis-elicited HIV-1-specific CD8+ T cells exhibited effector functions such as cytolysis and production of IFN- γ . The ability of recombinant, nonpathogenic rapidly growing mycobacteria to elicit antigen-specific CTL responses has never been reported previously. However, *M. tuberculosis* or recombinant *M. bovis* BCG have been shown to elicit antigen-specific CTL (Aldovini and Young, 1991; Kamath et al., 2004). The ability of HIV-1 vaccine vectors to elicit strong CTL responses is likely to be critical for vaccine-induced immune containment of HIV-1 replication and prevention of AIDS (Letvin, 2002).

Recombinant *M. smegmatis* was previously assessed as a vaccine in a mouse tumor model (Cheadle et al., 2005). Cheadle et al. showed that *M. smegmatis* was better than BCG at promoting DC maturation. However, recombinant *M. smegmatis* expressing a CTL epitope of the OVA antigen did not protect against challenge with a tumor expressing this epitope, whereas BCG expressing the same epitope protected mice against the OVA epitope-expressing tumor (Cheadle et al., 2005). This absence of anti-tumor activity elicited by recombinant *M. smegmatis* was associated with poor presentation of peptides by the non-pathogenic mycobacteria to an OVA-specific T cell line *in vitro* (Cheadle et al., 2005). However, since the OVA-specific T cell responses were not measured after immunization with the recombinant mycobacteria in this study, the inability of recombinant *M. smegmatis* to confer protection against a tumor challenge could not be attributed to inefficient induction of tumor antigen-specific CTL responses *in vivo*. In contrast to the findings of Cheadle et al. (2005) recombinant *M. smegmatis* was shown to access the MHC class I pathway better than BCG for presentation of peptide antigens (Neyrolles et al., 2001). Furthermore, a recombinant *M. smegmatis* expressing TNF- α was shown to have anti-tumor properties in mice (Young et al., 2004). The conflicting findings in these studies may be explained by the nature of the antigen expressed by mycobacteria. Neyrolles et al. (2001) expressed the influenza NP CTL epitope fused to the 19-kDa lipoprotein in mycobacteria. In contrast, Cheadle et al. (2005) expressed a secreted OVA CTL epitope in mycobacteria.

The kinetics of the *rM. smegmatis*-elicited T cell responses differed from those of T cell responses generated using other vaccine modalities. *rM. smegmatis*-elicited HIV-specific CD8+ T cell responses were maximal one week after immunization. This peak T cell response is earlier than responses elicited by plasmid DNA, adenoviral vectors and vaccinia vectors, which generally are maximal 10 to 14 days post-immunization (Barouch et al., 2003; Seaman et al., 2004). Interestingly, an early peak immune response has also been described in mice immunized with recombinant *Listeria monocytogenes* (Kaech and Ahmed (2001). *rM. smegmatis* also induced

peak T cell responses that were of lower magnitude than those induced by recombinant viral vectors, but similar in magnitude to those elicited by plasmid DNA (Seaman et al., 2004).

The maturation and differentiation status of the *rM. smegmatis*-elicited CD8+ T cells was defined using mAbs specific for CD44, CD62L, and CD127 (Huster et al., 2004). In both the peripheral blood and spleen of *rM. smegmatis*-immunized mice, the majority of the HIV-specific CTL generated were effector cells (CD44^{hi}, CD127⁻, CD62L^{lo}) and very few effector memory (CD44^{hi}, CD127⁺, CD62L^{lo}) and central memory (CD44^{hi}, CD127⁺, CD62L^{lo}) CTL were seen at the time of peak immune responses. Interestingly, in mice receiving two immunizations with *rM. smegmatis*, we also found a small but stable population of HIV-1-specific central memory CD8+ T cells. These data therefore suggest that *rM. smegmatis* is capable of generating both HIV-1-specific effector and memory cells *in vivo*. The ability of the *rM. smegmatis* vector to generate central memory cells is particularly important since these cells have been shown to expand *in vivo* and mediate protective immunity following a challenge with a pathogenic organism (Wherry et al., 2003).

There is growing evidence that vector or pathogen persistence may have an adverse effect on the generation of T cell memory. Persistent LCMV and lentiviral infections result in the generation of T cells that have lost the ability to perform some important effector functions (Appay et al., 2000; Fuller and Zajac, 2003; McKay et al., 2002; Wherry et al., 2003; Zajac et al., 1998). Persistent mycobacterial infections by slow-growing *M. tuberculosis* and BCG may also adversely affect T cell memory responses. On the other hand, vectors that do not persist can generate good T cell memory (Wherry and Ahmed, 2004). Therefore, the rapidly growing *M. smegmatis* vector may be better at eliciting memory T cell responses than persistent mycobacterial vectors because *M. smegmatis* is eliminated rapidly in the host.

Interestingly, we found that the multi-copy and the single copy vectors elicited comparable tetramer responses despite the fact that the multi-copy *rM. smegmatis* construct expressed significantly more HIV-1 envelope protein. High levels of gp120 expression have been shown to be toxic to mycobacteria (Stover et al., 1993). Consistent with this finding, we observed that the *in vitro* growth of the multi-copy *rM. smegmatis* was slower than the single copy vector (data not shown). Moreover, studies have shown that recombinant mycobacteria containing an integrated HIV transgene stably express that transgene and are highly immunogenic (Mederle et al., 2002; Stover et al., 1991). Thus, recombinant mycobacteria with integrated transgenes appear to be useful vaccine vectors.

A major limitation of the clinical utility of a number of vaccine vectors currently in development is the inhibition of vector immunogenicity by pre-existing anti-vector immunity. For example, immunity to the HIV-1 vaccine vector adenovirus serotype 5 (rAd5) has been

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shown to blunt the immunogenicity of rAd5 vaccines (Barouch et al., 2004; Molnar-Kimber et al., 1998). Since BCG is administered to a large proportion of the human population as a TB vaccine, anti-mycobacterial immunity might diminish the immunogenicity of recombinant mycobacterial vectors such as *rM. smegmatis*. However, our data indicate that BCG immunity affects the immunogenicity of *rM. smegmatis* only modestly. Consistent with this observation, it was previously reported that mice immunized with wildtype BCG still developed T cell and antibody responses to the HIV-1 Nef and β -galactosidase transgenes expressed in recombinant BCG (Gheorghiu et al., 1994). Pre-existing immunologic memory responses to BCG could result in the rapid destruction of recombinant *M. smegmatis*, which might favor cross-priming of the heterologous HIV-1 gp120 antigen (Kaufmann and Schaible, 2005). Hence, recombinant mycobacterial vaccines may be useful in BCG-immunized individuals.

Supplemental data for these studies is provided in Tables 2 and 3 (below) and FIGS. 9-14.

Vectors	Designed for	Marker	Inserts	Cloning sites	Seq. confirmation
pMV261	Intracellular expression	Kanamycin	Con6-gp120 & Con6-gp140CF	<i>BamHI/HpaI</i>	Whole inserts: OK
pMV361	Chromosomal integrated vector	Kanamycin	Con6-gp120 & Con6-gp140CF	<i>EcoRI/HpaI</i>	Whole inserts: OK
pJH152	Surface expression	Kanamycin	Con6-gp120 & Con6-gp140CF	<i>ApaI/HpaI</i>	Junction region: OK
pJH153	Intracellular expression	Kanamycin	Con6-gp120 & Con6-gp140CF	<i>ApaI/HpaI</i>	Junction region: OK
pJH154	Secreted expression	Kanamycin	Con6-gp120 & Con6-gp140CF	<i>ApaI/HpaI</i>	Whole inserts: OK
pYUB2051	Intracellular expression	Hygromycin	Con6-gp120 & Con6-gp140CF	<i>ApaI/HpaI</i>	Junction region: OK
pYUB2052	Surface expression	Hygromycin	Con6-gp120 & Con6-gp140CF	<i>ApaI/HpaI</i>	Junction region: OK
pYUB2053	Secreted expression	Hygromycin	Con6-gp120 & Con6-gp140CF	<i>ApaI/HpaI</i>	Junction region: OK
pJH222	Surface expression	Kanamycin	Con6-gp140CF	<i>NdeI/SpeI</i>	Junction region: OK

Table 2. Summary of Mycobacterial expression plasmids expressing HIV-1 CON6-gp120 or CON6-gp140CF. This table shows a summary of mycobacterial plasmids expressing HIV-1 CON6-gp120 or CON6-gp140CF that have been made. pMV261 is a constitutive expression vector and pMV361 is a coincident increase in Hsp60-fusion protein in response to stress with heat, acid, and peroxide. pJH vectors have kanamycin resistant marker and designed to deliver expressed proteins in different localization: pJH152, surface expression; pJH153, intracellular expression; pJH154, secreted expression. HIV-1 envelopes also constructed in hygromycin resistant mycobacterial expression vectors such as pYUB2051, pYUB2052, and pYUB2053.

Ability of Recombinant <i>M. smegmatis</i> Expressing Group M Consensus Envelope gp120 and gp140CF to Prime for a Recombinant Group M Consensus Env gp140CF Protein Boost		
Prime X2	Boost X1	Anti HIV-1 gp140 Geometric Mean Titer (n= 5 mice)
<i>M. smeg</i> empty plasmid control	rgp140CF 50ug protein	609
<i>M. smeg</i> -pJH154 gp120 secreted	rgp140CF 50ug protein	7,211*
<i>M. smeg</i> -pJH154 gp140CF secreted	rgp140CF 50ug protein	4,497*
None	rgp140CF 50ug protein	758
Dose of <i>M. smegmatis</i> = 10 ⁸ or 10 ⁹ CFU. * = p<0.05 compared control		

Table 3. Summary of r-m. *smegmatis* immunization data. Table 3 shows a summary of end-point titers of mouse study groups. End-point titers are determined when OD E/C is equal to or greater than 3. Our results show that the immunization via intraperitoneal (IP) is better antibody reactivity than intradermal (ID) injection. Mice groups immunized by CON6-gp120 gave better antibody reactivity than those immunized by CON6-gp140CF. It also indicated that the expression of HIV ENV as a secreted antigen would be better (pJH154-gp120/*M. smegmatis*). Interestingly, there was no dose effect and the highest dose (10¹⁰ CFU) showed very low immune response. There were two controls in this study: one was the immunization of *M. smegmatis* harboring no mycobacterial plasmid/constructs and the other was of *M. smegmatis* harboring empty pJH222 plasmid. Comparing and statistical analysis between individual groups among intraperitoneal immunization is showed in the right column. For example, the comparison of group G2/G13 (*M. smegmatis* vs pJH153-gp120/*M. smegmatis* as 10⁹ CFU) is showed in the table as statistically significant (p<0.01). Another example was the comparison of G7/G18 (Empty pJH222/*M. smegmatis* vs pJH154-gp120/*M. smegmatis* as 10⁷ CFU), which was statistically significant (0.01<p<0.02). This data were used to establish that the mouse immunization study of BCG strains will use intraperitoneal injection and immunization with moderate dosage. It showed pJH154, which designed to deliver target protein as a secreted antigen, is a better choice to express HIV envelopes in BCG.

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Example 3. Further optimization of expression of *P. falciparum* MSP antigen.

The XbaI/BamHI fragment, which contains the α -antigen promoter, 19 kD signal sequence and msp1.19 (Example 1) was amplified from pJH222 and cloned in pMV261 replacing the XbaI/BamHI fragment. Fragment 1 or Fragment 2 of msp1.42 (FIG. 16) were cloned in the vector replacing msp1.19. The final construct was transformed into *Mycobacterium smegmatis*, mc² 155 and protein lysate from 1 ml of culture was extracted and analyzed by SDS-PAGE and Western. Protein bands specific to monoclonal antibody *Plasmodium falciparum*,

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mAb 5.2 (MRA-94, Malaria Research and Reference Reagent Resource Center) were visualized after incubation with a secondary antibody, (NA 931V, Amersham Biosciences; anti-mouse IgG linked to horse radish peroxidase). As shown in FIG. 16, Fragment 1, containing most of the MSP 1.33 clone, exhibited much greater expression of the MSP antigen.

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Example 4. Further characterization of *M. smegmatis* expressing the HIV-1 gp120 antigen

rM. smegmatis elicited HIV-1 Env-specific CD4⁺ T cells in mice. To determine the ability of *M. smegmatis* vector and other vaccine vectors to elicit gp120-specific CD4⁺-T-cell responses, splenocytes from immunized mice were exposed *in vitro* to a pool of peptides spanning the HIV-1 gp120 protein. As shown in FIG. 17, immunization of mice with Smeg-gp120 elicited HIV-1-specific CD4⁺ T cells that produced levels of IFN- γ comparable to those produced by CD4⁺ T cells from Vaccinia-gp160 or DNA-gp120 immunized mice, and higher than those produced by CD4⁺ T cells from mice immunized with Ad-gp140. IL-2 production by gp120-specific CD4⁺ T cells was comparable in Smeg-gp120 and Vac-gp160 immunized mice and increased following boosting. Interestingly, production of IL-2 by gp120-specific CD4⁺ T cells of DNA-gp120 immunized mice was the highest at the time of the peak immune responses following the prime and boost immunization, but did not appear to be durable, since it decreased by week 10 following the priming immunization. As in the case of IFN- γ production, gp120-specific CD4⁺ T cells of mice immunized with Ad-gp140 produced the lowest IL-2 levels. These findings indicate that Smeg-gp120 immunized mice develop Th1-type immune responses that rapidly gain memory function.

rM. smegmatis vector efficiently primed T cell responses in a prime/boost immunization strategy. Although the frequency of p18-specific splenic CD8⁺ T cells in the *rM. smegmatis* immunized mice was not as high as that seen in mice that received the other immunogens, the phenotypic evidence that these cells were predominantly memory T lymphocytes encouraged us to evaluate *M. smegmatis* as a priming vector in a heterologous immunization regimen. Since plasmid DNA is currently used as the primary vaccine modality in many prime/boost vaccine regimens in advanced clinical trials, we chose to compare *rM. smegmatis* with plasmid DNA in this study. Groups of mice were injected a single time with Smeg-gp120 or DNA-gp120 and 20 weeks later the mice were boosted with a suboptimal dose (10^6 particles) of Ad-gp140. Other groups of mice were injected twice with Smeg-gp120 or DNA-gp120, 10 weeks apart, and 10 weeks later boosted with Ad-gp140. FIG. 18A shows the phenotypic profile of the p18-specific CD8⁺ T cells in the peripheral blood of each group of immunized mice on the day of the Ad-gp140 boost. In mice primed with Smeg-gp120, more than 93% of the p18-specific CD8⁺ T cells expressed CD62L and CD27, surface molecule associated with T cell memory function, while in

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DNA-gp120 primed mice only 50% of the p18-specific CD8⁺ T-cell expressed CD62L and 70% expressed CD27. The differences between the phenotypic profiles of the p18-specific CD8⁺-T-cell elicited by these two vectors were more marked after two immunizations. In the Smeg-gp120 immunized mice 85% and 92% of these epitope-specific cells expressed CD62L and CD27, respectively, while in the DNA-immunized mice only 15% and 35% of p18-specific CD8⁺ T cells expressed CD62L and CD27, respectively. No significant differences were observed in the expression of CD127 by p18-specific CD8⁺ T-cells in the Smeg-gp120 and DNA-gp120 immunized mice. The Smeg-gp120 and DNA-gp120 immunized mice also differed in the level of p18-specific CD8⁺ T cells seen in their peripheral blood. On the day of Ad-gp140 boosting, the Smeg-gp120 and DNA-gp120 immunized mice demonstrated 0.05% and 0.5% p18-specific CD8⁺ T cells, respectively, and the second immunization increased these percentages to 0.2% and 4.0%, respectively. Thus, although the Smeg-gp120 immunized mice had gp120-specific CD8⁺ T lymphocytes that were predominantly memory cells, the tetramer-positive cells were a much smaller percent of the CD8⁺ T cell population in these mice.

The mice were then inoculated with a suboptimal dose of Ad-gp140 (10⁶ particles) and the kinetics of the generation of p18-specific CD8⁺ T cells were assessed. The use of a sub-optimal dose of Ad-gp120 was chosen to facilitate discrimination between the priming efficiency of plasmid DNA and rM. *smegmatis* immunogens. One week after the Ad-gp140 immunization, the DNA-immunized mice (one or two immunizations) had generated higher p18-specific CD8⁺ T cell responses than the Smeg-gp120 immunized mice (FIG. 8B). However, by the second and third weeks post-immunization, Smeg-gp120 immunized mice had comparable p18-specific CD8⁺ T cell responses. The contraction phase of Smeg-gp120 and DNA-gp120 immunized mice was similar as well and the magnitude of the p18-specific CD8⁺ T cells was equal for long time. Therefore, in spite having a low percentage of p18-specific CD8⁺ T cells, the Smeg-gp120 immunized mice developed robust secondary CD8⁺ T cell responses specific for this viral epitope.

Heterologous recombinant *M. smegmatis* prime/recombinant Adenovirus vector boost immunization elicited IL-2 and IFN- γ secreting HIV-1 specific CD4⁺ and CD8⁺ T cells. The functional properties of the antigen-specific CD4⁺ and CD8⁺ T cell responses generated in mice that received a recombinant *M. smegmatis* prime followed by a recombinant adenovirus boost was next evaluated. Priming of mice with either Smeg-gp120 or DNA-gp120 followed by Ad-gp140 boosting elicited higher frequency of HIV-1 p18-specific CD8⁺ T cells that secreted IFN- γ and were cytotoxic (measured by expression of CD107a and CD107b) compared to immunization with Ad-gp140 alone (FIGS. 19 and 20). The heterologous prime/boost immunization also generated HIV-1 specific CD4⁺ T cells that secreted IFN- γ and IL-2. Furthermore, more IFN- γ -producing CD4⁺ T cells were elicited in mice after two immunizations with Smeg-gp120 or DNA-

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gp120 than with a single immunization with either vector (FIG. 20B). Altogether, these data suggest that recombinant *M. smegmatis* can prime functional antigen-specific CD4+ and CD8+ T cell responses as efficiently as plasmid DNA.

5 In view of the above, it will be seen that the several advantages of the invention are achieved and other advantages attained.

 As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in
10 a limiting sense.

 All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by the authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinence of the cited references.
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What is claimed is:

1. A recombinant mycobacterium expressing an HIV-1 antigen and a malarial antigen.
2. The recombinant mycobacterium of claim 1, wherein the mycobacterium is
5 *Mycobacterium smegmatis*, *Mycobacterium bovis*-BCG, *Mycobacterium avium*, *Mycobacterium phlei*, *Mycobacterium fortuitum*, *Mycobacterium lufu*, *Mycobacterium paratuberculosis*, *Mycobacterium habana*, *Mycobacterium microti*, *Mycobacterium scrofulaceiu*, *Mycobacterium intercellulare*, *Mycobacterium tuberculosis*, or any genetic variant thereof.
- 10 3. The recombinant mycobacterium of claim 1, wherein the mycobacterium is *Mycobacterium smegmatis* or *Mycobacterium bovis*-BCG.
4. The recombinant mycobacterium of claim 1, wherein the mycobacterium is *Mycobacterium smegmatis* MC²155 or a derivative thereof.
15
5. The mycobacterium of claim 1, wherein the HIV-1 antigen is gp120 env, gp140 env, gp160 env, gag, pol, vif, vpr, vpu, tat, rev or nef.
6. The mycobacterium of claim 1, wherein the HIV-1 antigen is gp120 env.
20
7. The mycobacterium of claim 1, wherein the malarial antigen is MSP-1, AMA-1, or CSP-1.
8. The mycobacterium of claim 7, wherein the malarial antigen is PfMSP1-19.
25
9. The mycobacterium of claim 1, wherein the malarial antigen is expressed from a gene that has been engineered to have an increased GC content.
10. The mycobacterium of claim 1, further comprising a recombinant DNA sequence
30 encoding a detectable moiety.
11. The mycobacterium of claim 10, wherein the recombinant DNA sequence encodes a fluorescent protein or an antigen.

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12. The mycobacterium of claim 1, wherein the HIV-1 antigen and malarial antigen are expressed from genes under the control of a mycobacterial promoter, wherein the mycobacterial promoter for the HIV-1 antigen and the malarial antigen are independently *hsp60*, *mtrA*, *18kD*, α -*Ag*, or *aceA*.

5

13. The mycobacterium of claim 12, wherein the mycobacterial promoter for the HIV-1 antigen and the malarial antigen is α -*Ag*.

14. The mycobacterium of claim 1, wherein the HIV-1 antigen and the malarial antigen is expressed from genes further encoding a signal sequence that facilitates expression of the viral protein in the mycobacterial membrane.

10

15. The mycobacterium of claim 14, wherein the signal sequence is the 19-kDa signal sequence.

15

16. The mycobacterium of claim 1, wherein
the mycobacterium is *M. smegmatis* MC²155 or a derivative thereof;
the HIV-1 antigen is gp120 env;
the malarial antigen is MSP-1;
the HIV-1 antigen and the malarial antigen is expressed from genes further
encoding the 19-kDa signal sequence; and
the mycobacterial promoter for the HIV-1 antigen and the malarial antigen is α -*Ag*.

20

17. A *Mycobacterium smegmatis* expressing an HIV-1 antigen.

25

18. The *Mycobacterium smegmatis* of claim 17, which is *M. smegmatis* MC²155 or a derivative thereof.

19. The *Mycobacterium smegmatis* of claim 17, wherein the HIV-1 antigen is gp120 env, gp140 env, gp160 env, gag, pol, vif, vpr, vpu, tat, rev or nef.

30

20. The mycobacterium of claim 17, wherein the HIV-1 antigen is gp120 env.

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21. The *Mycobacterium smegmatis* of claim 17, wherein the HIV-1 antigen is expressed from a gene further encoding the 19-kDa signal sequence.

5 22. The *Mycobacterium smegmatis* of claim 17, wherein a mycobacterial promoter for the HIV-1 antigen is α -Ag.

23. The *Mycobacterium smegmatis* of claim 17, wherein
the mycobacterium is *M. smegmatis* MC²155 or a derivative thereof;
the HIV-1 antigen is gp120 env;
10 the HIV-1 antigen is expressed from a gene further encoding the 19-kDa signal sequence; and
the mycobacterial promoter for the HIV-1 antigen is α -Ag.

24. A vaccine comprising the recombinant mycobacterium of any one of claims 1-16,
15 wherein the mycobacterium is capable of inducing an immune response in a mammal against HIV-1 and the malarial pathogen.

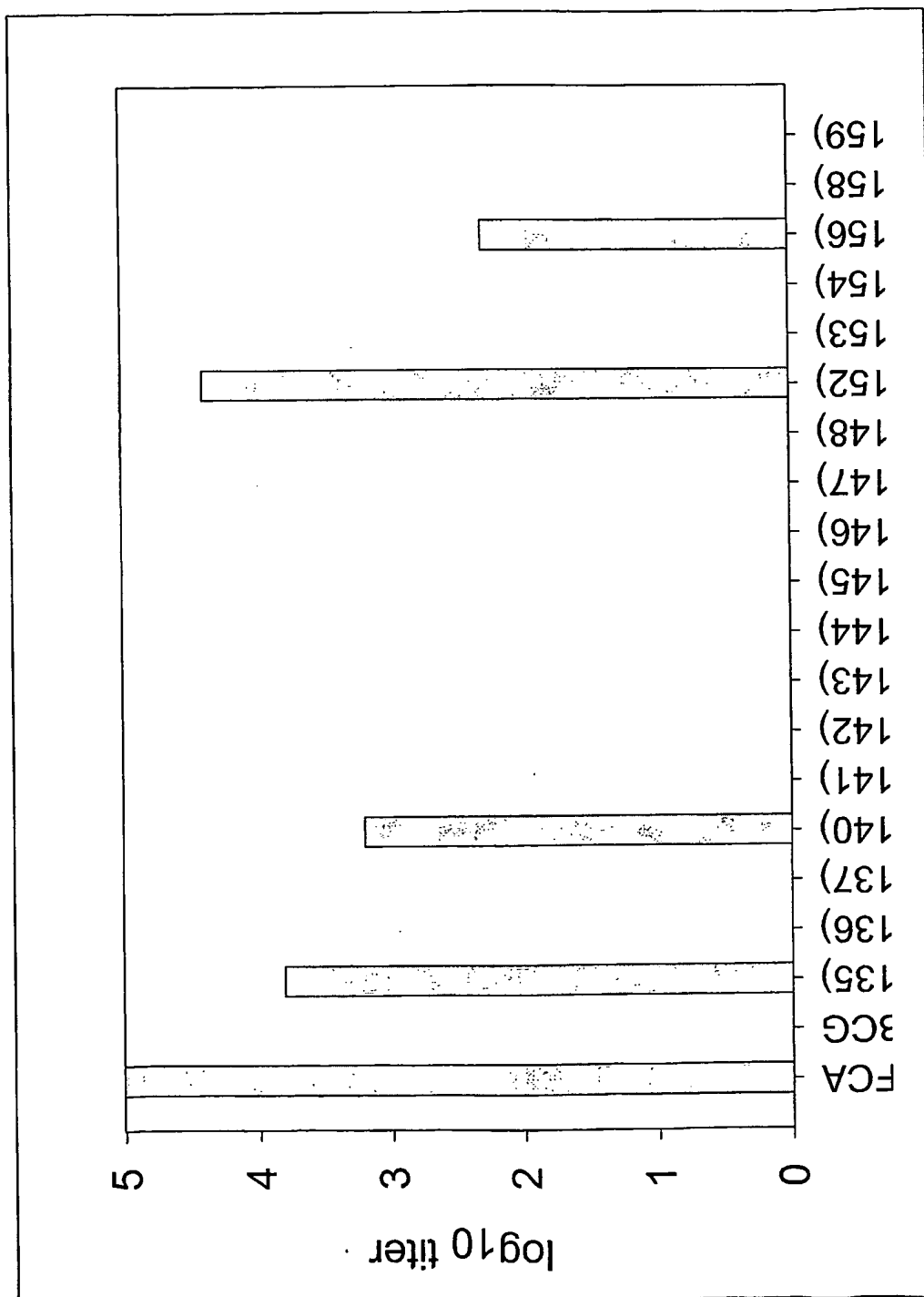
25. The vaccine of claim 24, wherein the mammal is a human.

20 26. A method of inducing an immune response in a mammal against HIV-1 and a malarial pathogen, the method comprising infecting the mammal with the mycobacterium of any one of claims 1-16.

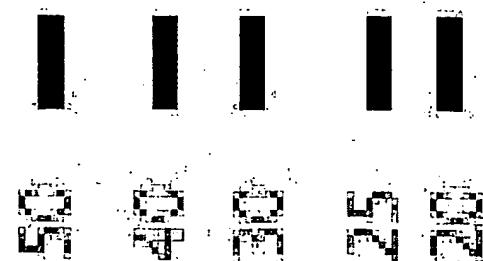
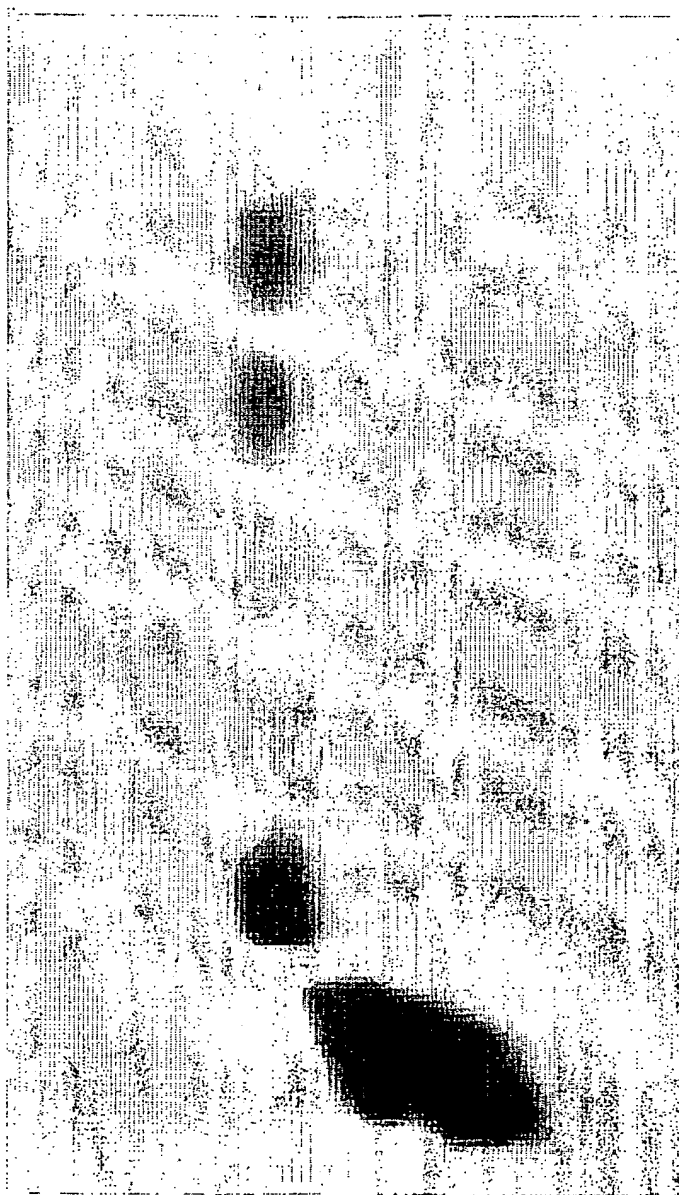
27. The method of claim 26, wherein the mammal is a human.

25 28. A method of inducing an immune response in a mammal against HIV-1, the method comprising infecting the mammal with the mycobacterium of any one of claims 1-23.

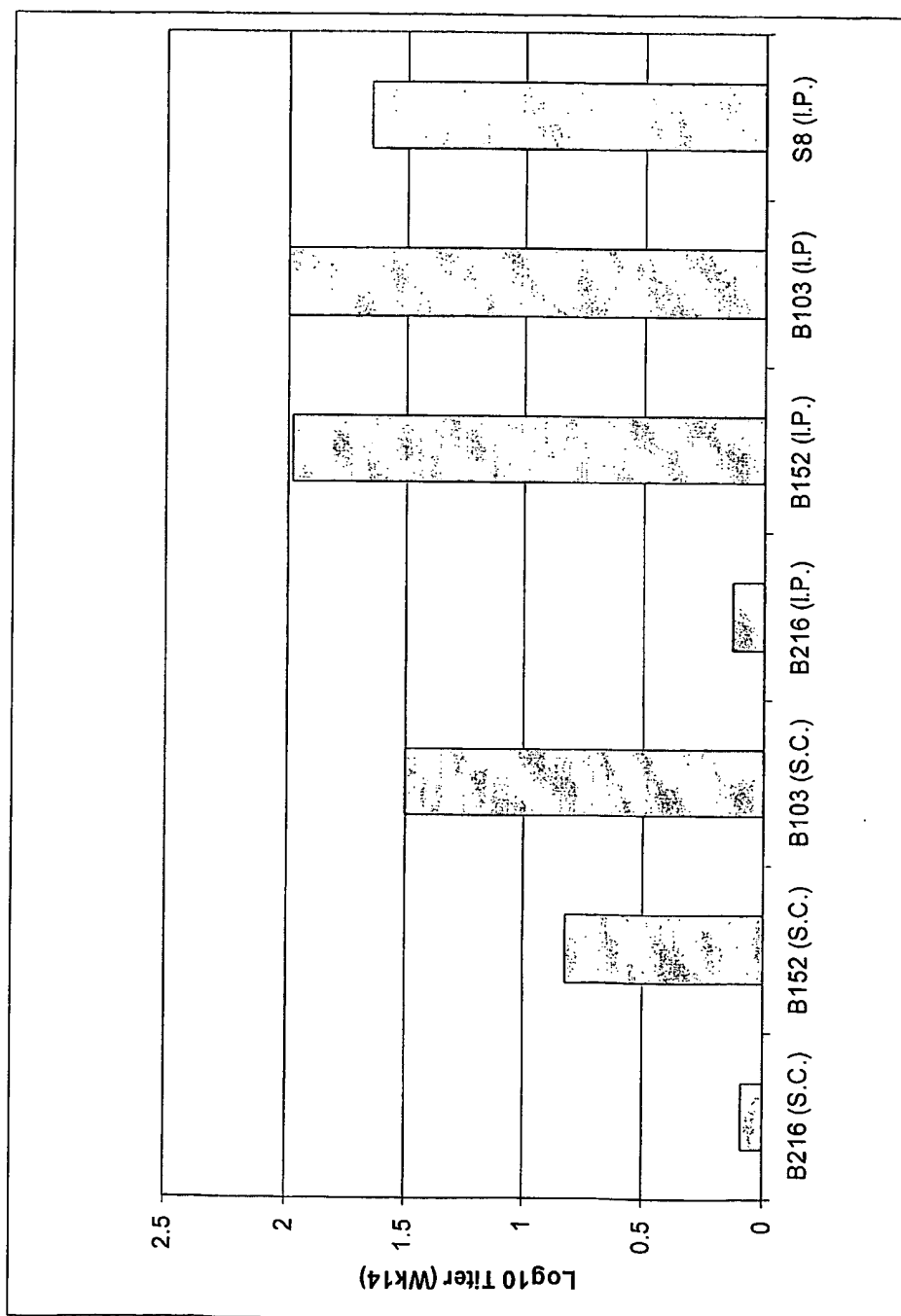
1/21
FIG. 1



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FIG. 2

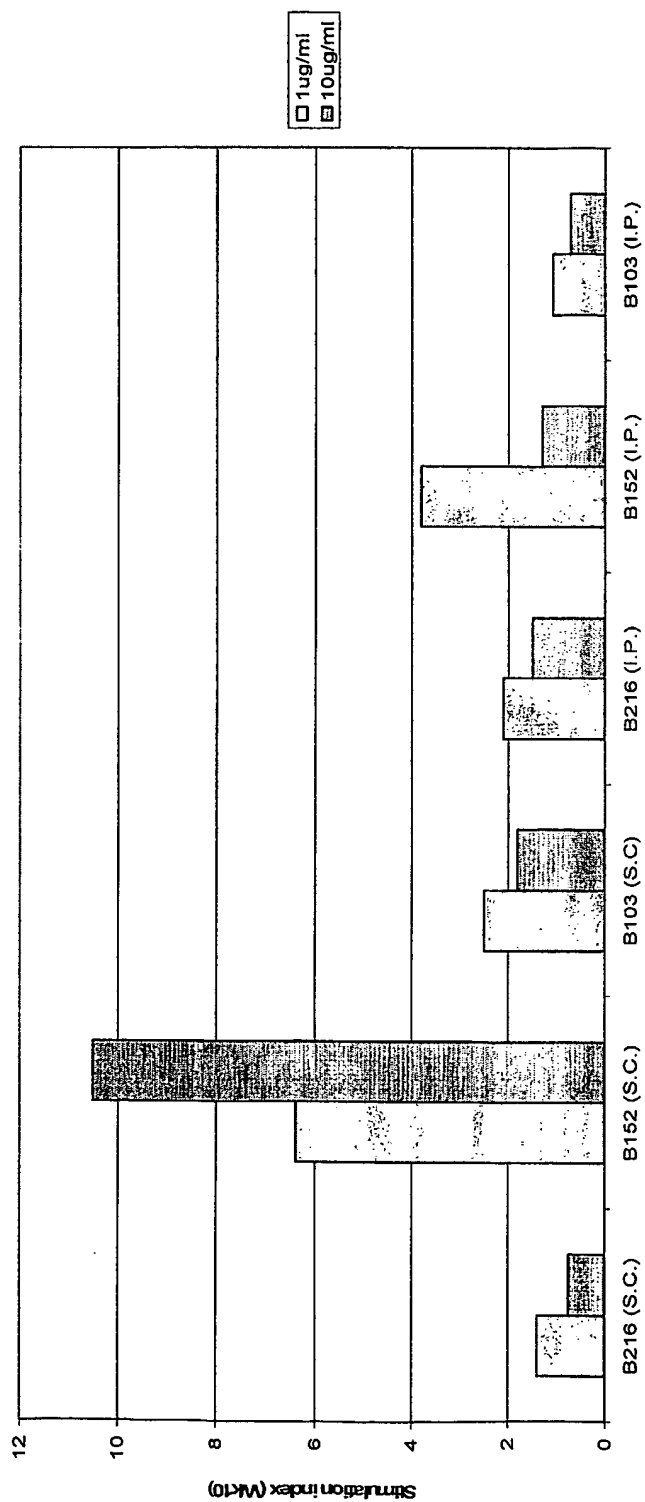


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FIG. 3

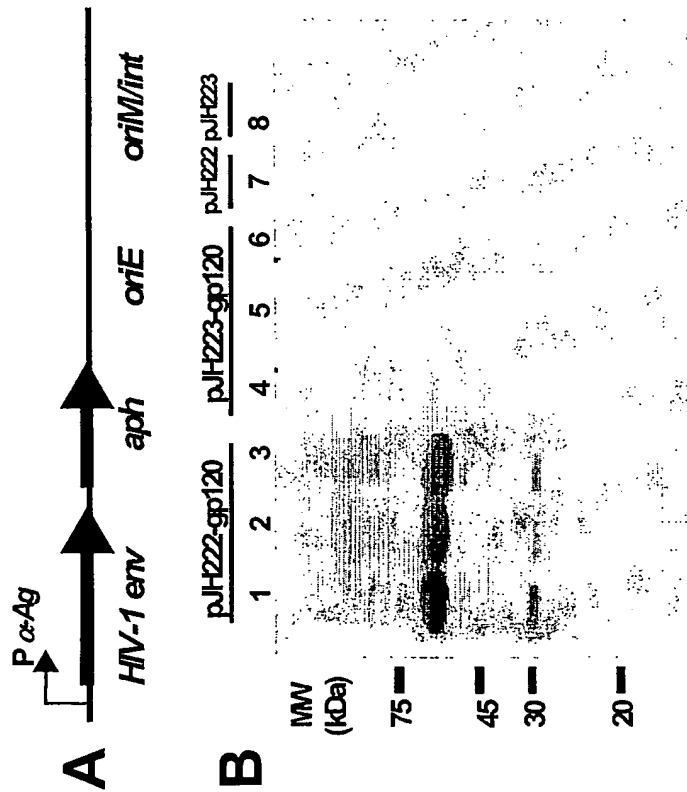


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FIG. 4

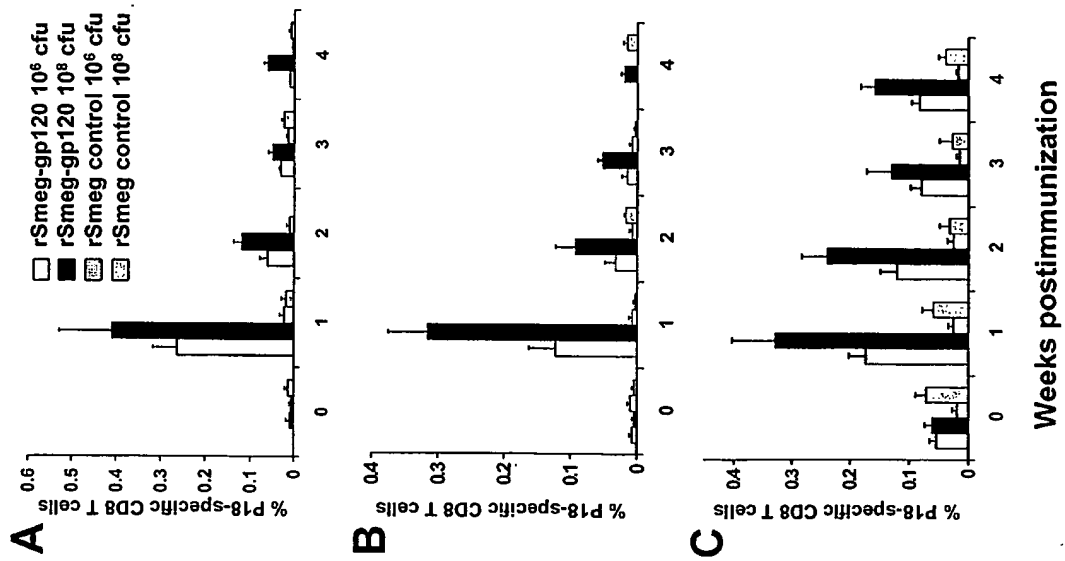


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FIG. 5

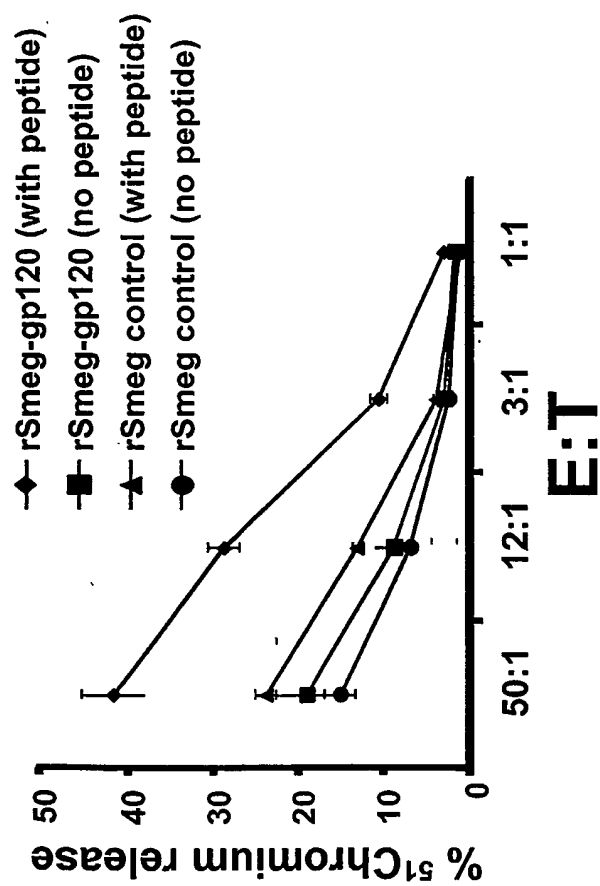


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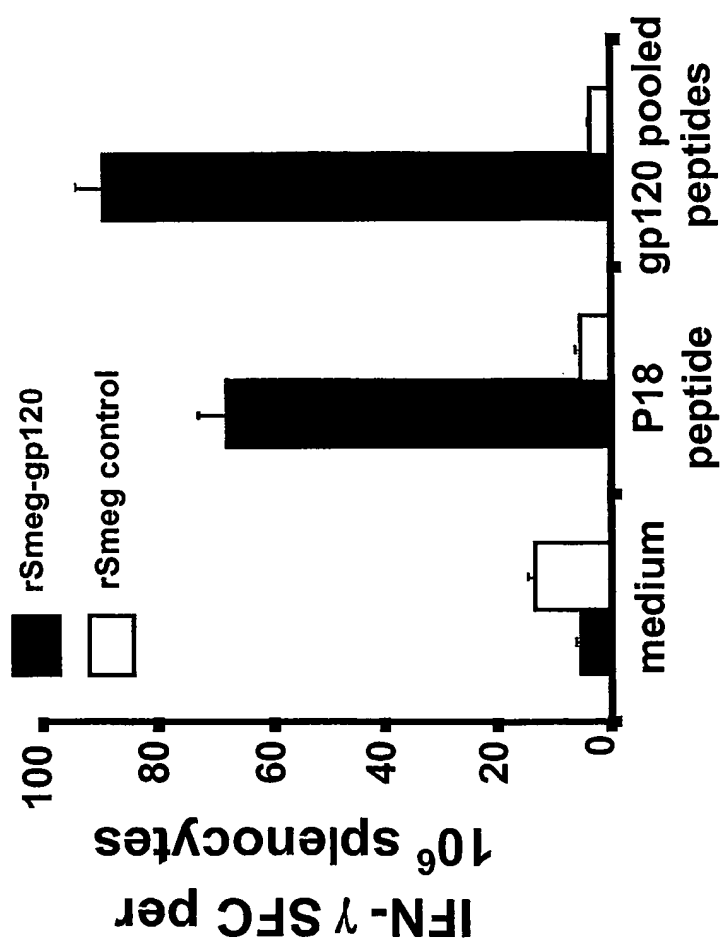
FIG. 6



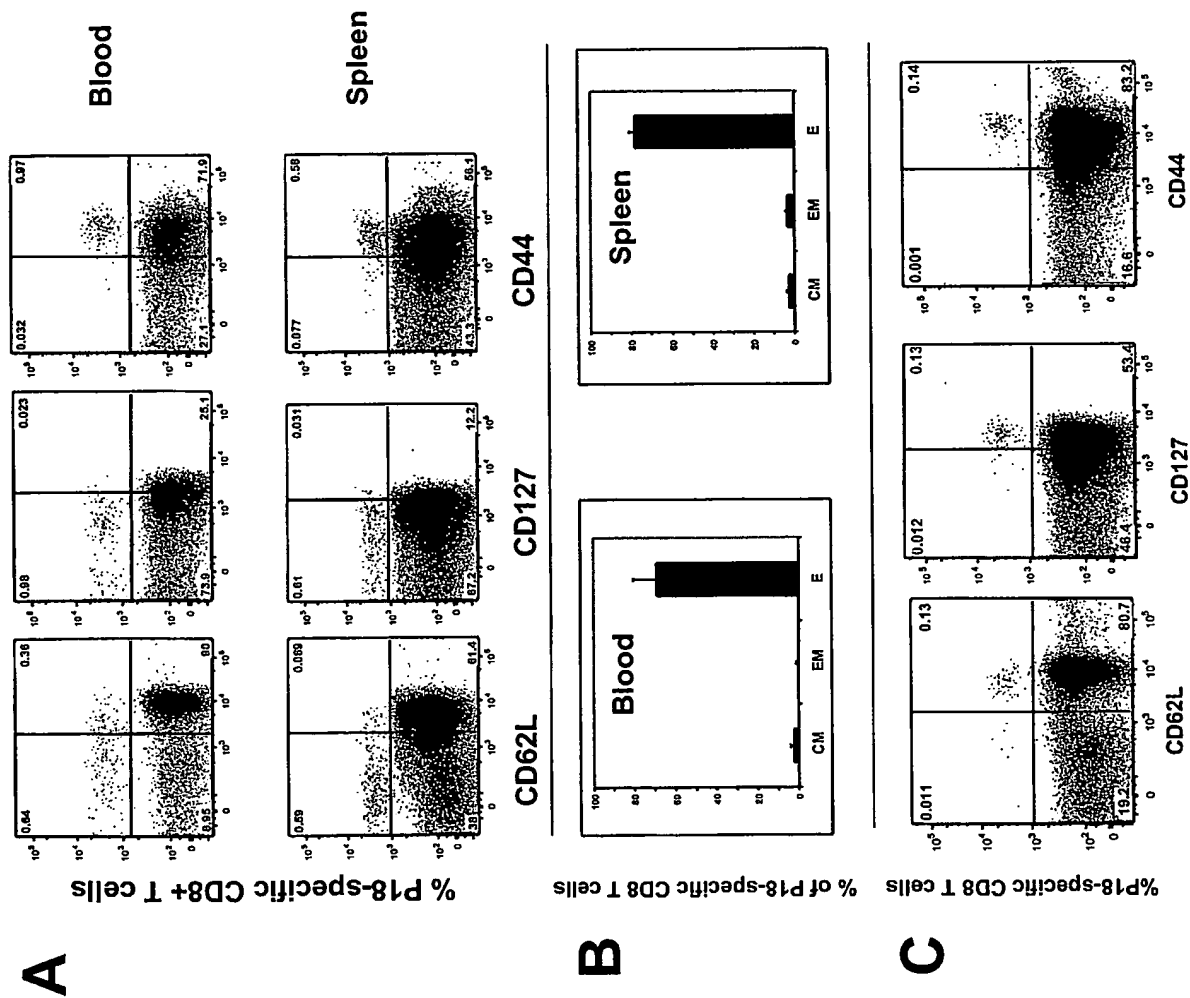
7/21
FIG. 7A



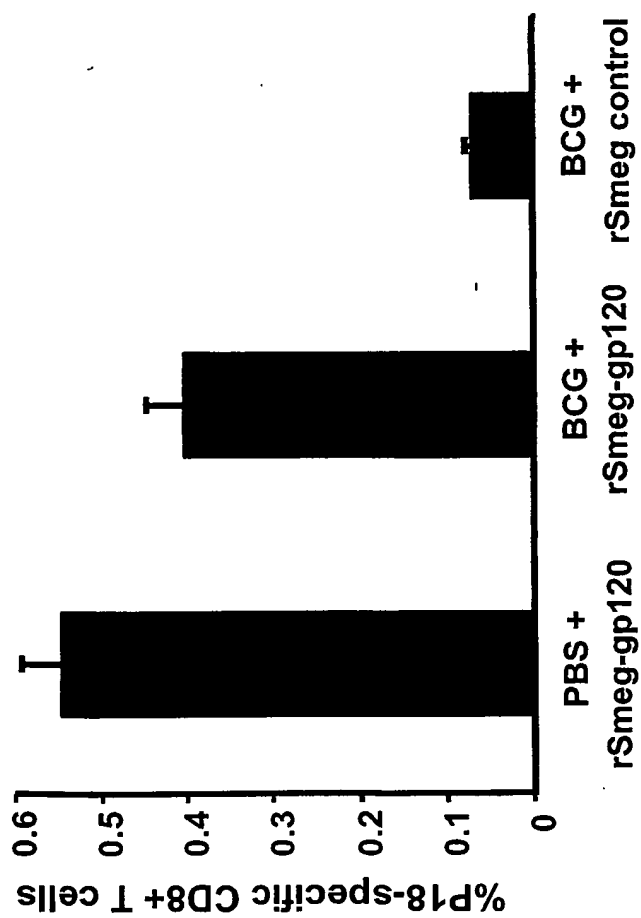
8/21
FIG. 7B



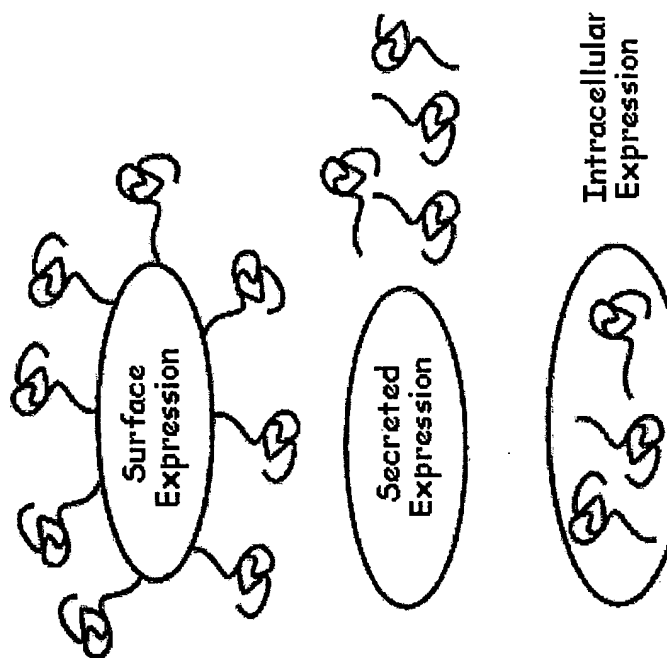
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FIG. 8



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FIG. 9

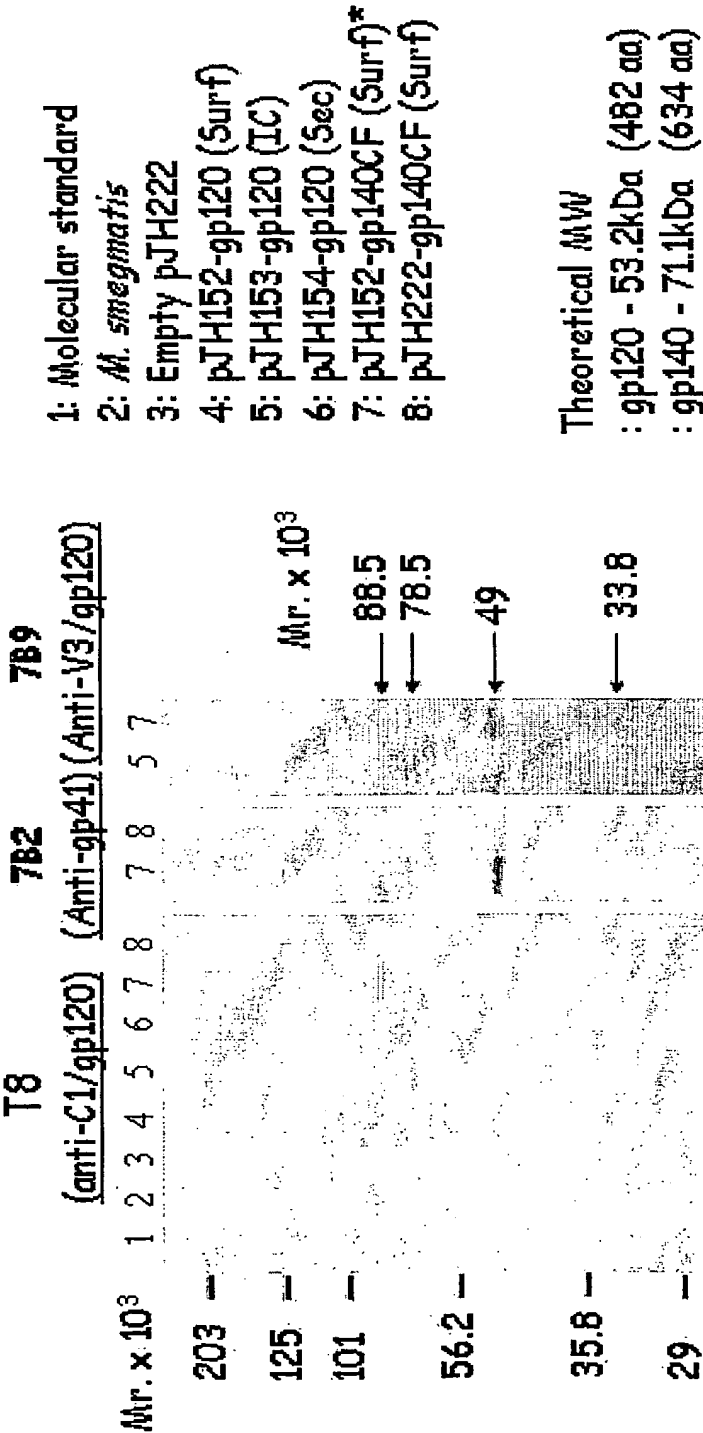


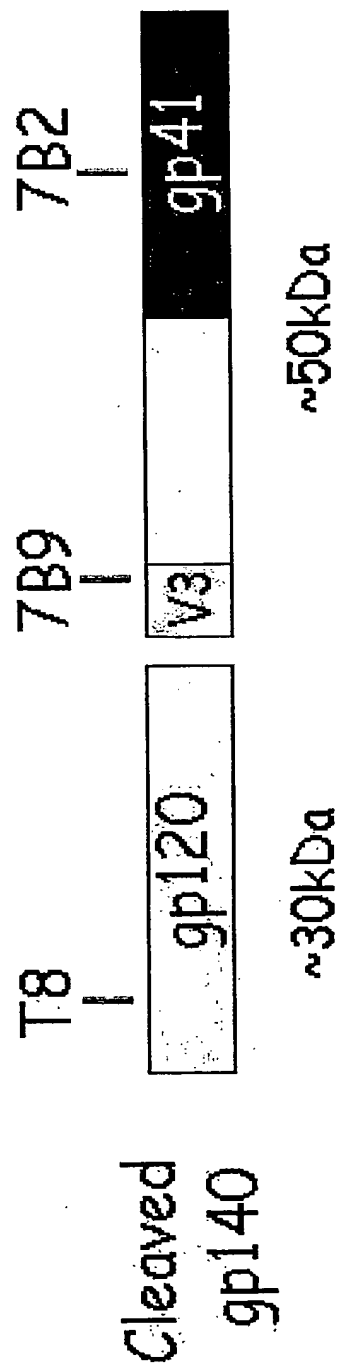
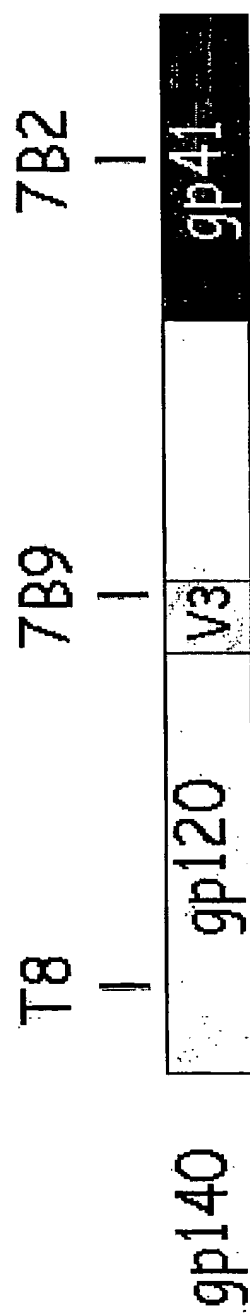
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FIG. 10



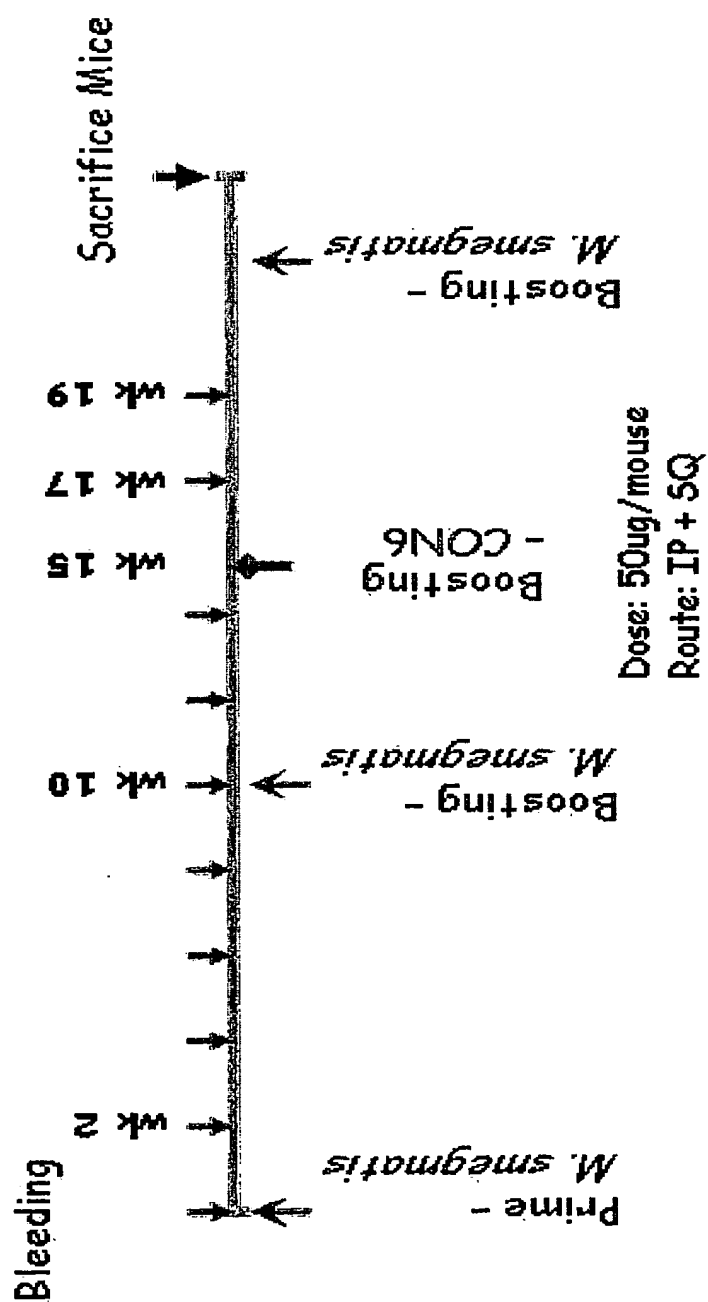
1. As a surface antigen.
2. As a secreted antigen.
3. As an intracellular expressed antigen.

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FIG. 11



























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FIG. 12

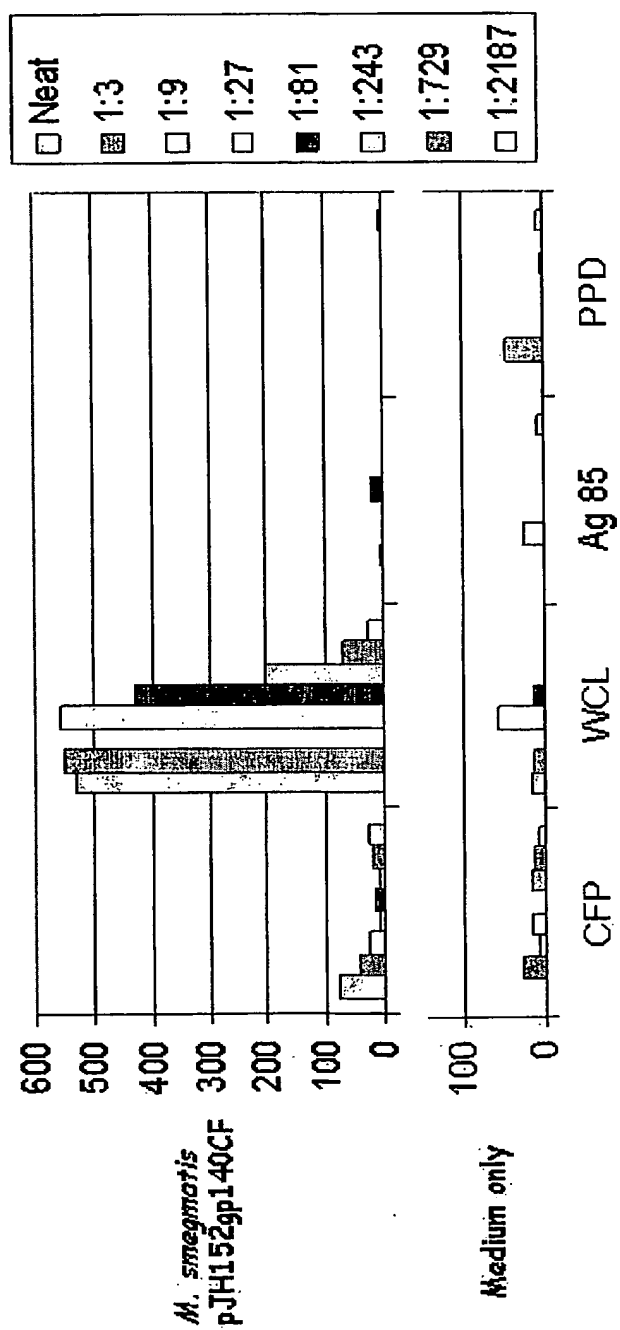
14/21
FIG. 13



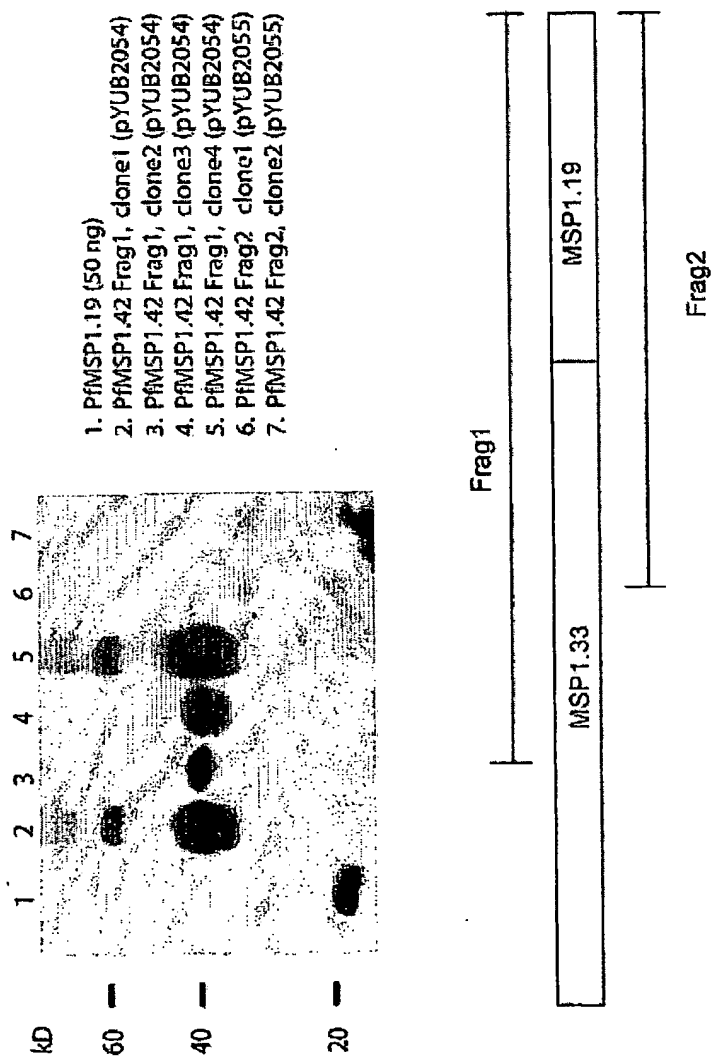
15/21
FIG. 14

Lysate Dilution	Ag	Ag	No Ag	Splenocyte number
Neat				2.5×10^5
Neat				1.25×10^5
1:3				2.5×10^5
1:3				1.25×10^5
1:9				2.5×10^5
1:9				1.25×10^5
1:27				2.5×10^5
1:27				1.25×10^5

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FIG. 15

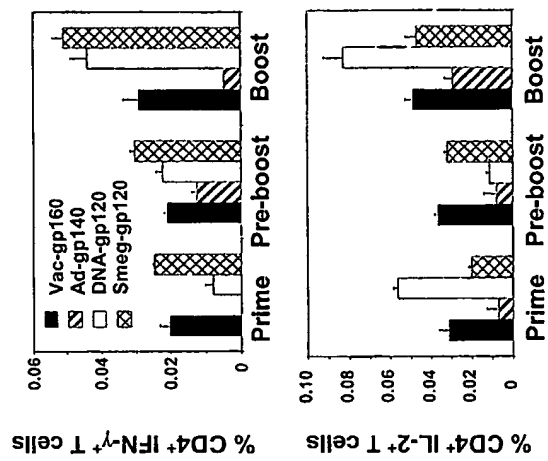


17/21 FIG. 16



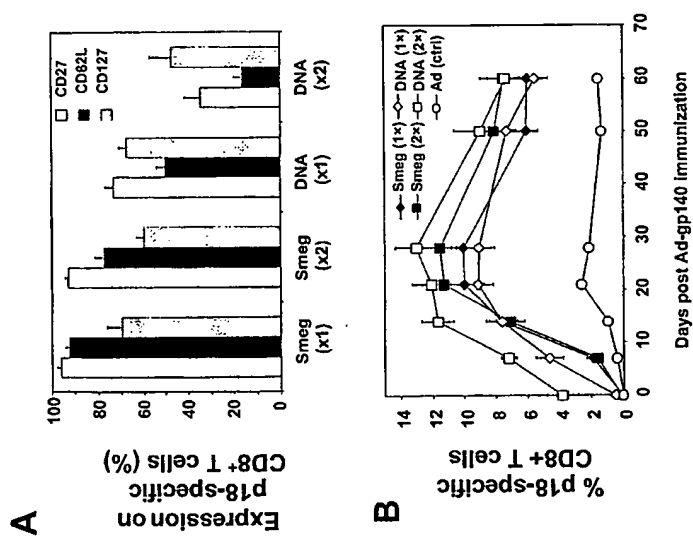
18/21

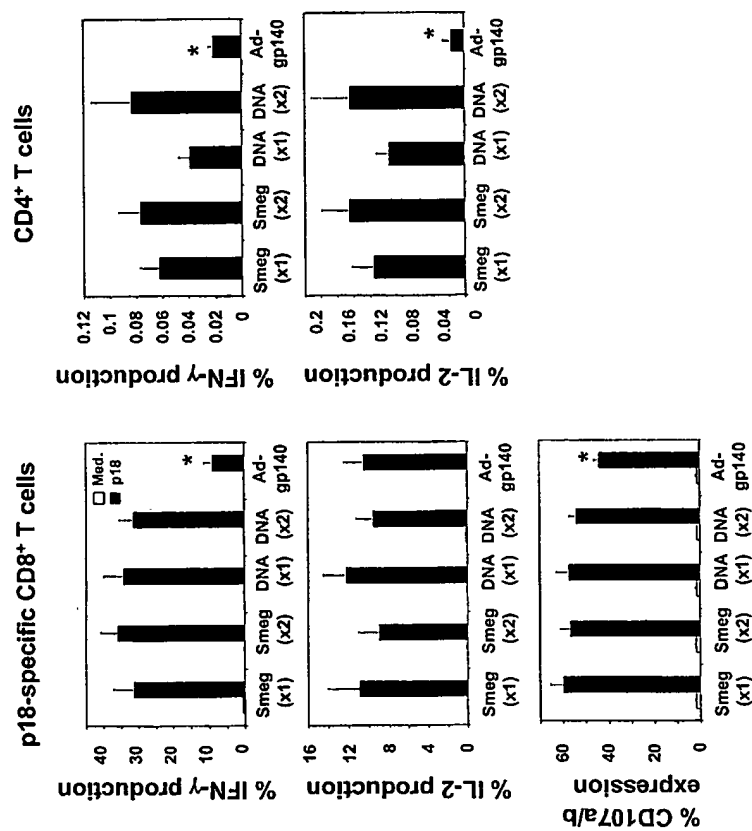
FIG. 17



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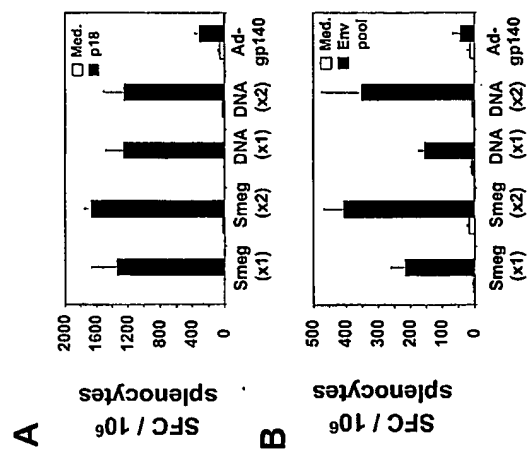
FIG. 18



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FIG. 19

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FIG. 20



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US06/00790

A. CLASSIFICATION OF SUBJECT MATTER

IPC: A61K 49/00(2006.01),39/00(2006.01),39/12(2006.01),39/04(2006.01);A01N 63/00(2006.01),65/00(2006.01);C12P 1/00(2006.01),21/04(2006.01);C12N 1/00(2006.01),1/12(2006.01),1/20(2006.01),7/00(2006.01),7/01(2006.01)

USPC: 424/9.1,9.2,93.1,93.2,93.4,184.1,204.1,248.1;435/41,71.1,235.1,243,253.1,440,471,473,863,866

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/9.1, 9.2, 93.1, 93.2, 93.4, 184.1, 204.1, 248.1; 435/41, 71.1, 235.1, 243, 253.1, 440, 471, 473, 863, 866

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
BIOSIS CABA CAPLUS EMBASE JAPIO LIFESCI MEDLINE SCISEARCH USPATFULL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6,074,866 A (ESCUYER et al) 13 June 2000, entire reference.	1-28
Y	US 6,472,213 B1 (ESCUYER et al) 29 October 2002, entire reference.	1-28
Y	US 5,736,367 A (HAUN et al) 07 April 1998, entire reference.	1-28
Y	US 6,423,545 B1 (PAVELKA, JR. et al) 23 July 2002, entire reference.	1-28
Y	US 5,773,267 A (JACOBS et al) 30 June 1998, entire reference.	1-28
Y	US 5,776,465 A (ODONNELL et al) 07 July 1998, entire reference.	1-28
Y	US 5,830,475 A (ALDOVINI et al) 03 November 1998, entire reference.	1-28

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search

08 May 2006 (08.05.2006)

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US06/00790

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GALLO, R.C. The end or the beginning of the drive to an HIV-preventive vaccine: a view from over 20 years. Lancet. 2005, Vol. 366, pages 1894-1898. Entire reference.	1-28
A	GILBERT, P.B. et al. HIV-1 virologic and immunologic progression and initiation of antiretroviral therapy among HIV-1-infected subjects in a trial of the efficacy of recombinant glycoprotein 120 vaccine. The Journal of Infectious Diseases. 2005, Vol. 192, pages 974-983, entire reference.	1-28
Y	US 6,372,478 B1 (BLOOM et al) 16 April 2002, entire reference.	1-28
X	US 6,235,518 B1 (GICQUEL et al) 22 May 2001, entire reference.	17-19,28
Y		1-16,20-27